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# Biochemistry of the Wood-rotting Fungi

## 4. METABOLIC PRODUCTS OF *TRAMETES SUAVEOLENS* (LINN.) FR.

By J. H. BIRKINSHAW, A. BRACKEN and W. P. K. FINDLAY, *Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London, and The Forest Products Research Laboratory, Department of Scientific and Industrial Research*

(*Received* 15 *February* 1944)

In an examination of the volatile metabolic products of the odoriferous fungus *Lentinus lepideus* Fr., Birkinshaw & Findlay (1940) identified the methyl esters of *p*-methoxycinnamic, and cinnamic acids. Another acid, anisic acid, was also present as an ester but the nature of the alcohol moiety was left undetermined, although it was considered that the ester was probably the methyl ester.

Another wood-rotting fungus which possesses a characteristic odour is *Trametes suaveolens* (Linn.) Fr. This fungus is frequently responsible for decay in willow trees. The odour of the natural fruit body is recorded by Ramsbottom (1923) as 'strong of anise', that of the mycelium in culture is described by Badcock (1939) as 'strong, almost anisaldehyde'. The identification of the volatile products of this fungus forms the subject of the present communication.

The fungus was grown on a liquid malt extract medium, and the volatile products in both the mycelium and the culture fluid were examined. The main product from both sources was methyl anisate (methyl *p*-methoxybenzoate). The odour of methyl anisate recalls that of *T. suaveolens* in culture, and this product is no doubt partly responsible for the smell produced by the fungus. The occurrence of anisic acid as its methyl ester in the products of *T. suaveolens* strengthens the probability that it is also combined in this form in the products of *Lentinus lepideus*. In addition to methyl anisate small amounts of anisaldehyde (*p*-methoxybenzaldehyde) and of free anisic acid (*p*-methoxybenzoic acid) were detected. Anisaldehyde is a constituent of many ethereal oils derived from higher plants, particularly of those containing anethole, from which it is produced by atmospheric oxidation. It occurs in oil of anise which *Trametes suaveolens* resembles in odour. The presence of small amounts of free anisic acid is to be expected, since anisaldehyde is readily oxidized to anisic acid when exposed to the air. The absence of appreciable amounts of cinnamic acid derivatives is of interest, since these were the major products isolated from *Lentinus lepideus*.

## EXPERIMENTAL

*Organism.* The culture of *Trametes suaveolens* (Linn.) Fr. used was isolated from a sporophore of the fungus found growing on a willow tree near Oxford.

*Method of culture.* The fungus was grown on a 5% aqueous solution of commercial malt extract distributed in 95 one-litre conical flasks each containing 350 ml. of the medium. The flasks were sterilized and inoculated with small portions of mycelium of *T. suaveolens* derived from malt-agar slopes; they were then incubated at 24°. The cultures were harvested after 34 days.

### Examination of metabolic products

#### *Mycelium*

This was separated from the culture fluid by filtration and subjected to steam distillation. About 5 l. of distillate were collected. It contained crystals (plates) which were increased in amount by chilling. These were collected and dried; 0·4 g., m.p. 48–48·5°. They were shown by analysis and by their failure to depress the m.p. of an authentic specimen of m.p. 48·5–49° to be methyl anisate. (Found: C, 65·2; H, 6·07; MeO, 37·1. Calc. for $C_9H_{10}O_3$: C, 65·04; H, 6·06; 2MeO, 37·35%.)

The filtrate from the steam distillate was extracted with ether. After removal of the ether the residue (0·86 g.) was partly crystalline. The crystals, freed from adherent oil by pressing on porous tile, weighed 0·3 g. and had m.p. 47–48°. They consisted of slightly impure methyl anisate.

#### *Culture filtrate*

The filtrate was extracted with 6 l. of ether. The extract (8·0 g.) was an oil containing crystals. After pressing on porous tile, the crystals weighed 1·47 g. and had m.p. 40–43°. These also proved to be impure methyl anisate.

The tile was ground up, together with that containing residues from the steam distillate of the mycelium, and extracted with ether. The total oil (6·81 g.), containing some crystals, was distilled *in vacuo*. The main fraction (Fraction I), 3·73 g., b.p. 123–125°/5 mm., was at first a colourless oil but later it crystallized. A small second fraction (Fraction II) was obtained of wt. 0·64 g., b.p. 140–175°/about 1 mm. There was an undistilled residue (2·01 g.) which showed signs of incipient decomposition, and was not further examined.

*Treatment of distillates from ether extract of culture filtrate*

(*a*) *Fraction I* (3·73 g., b.p. 123–125°/5 mm.). This distillate was at first an oil, but on touching the surface with a glass rod it set to a mass of crystals, with some oil. It was pressed on porous tile and gave 1·9 g. of crystals of m.p. 46·5–47·5°, consisting of impure methyl anisate. The oil (1·7 g.) was recovered from the porous tile by ether extraction and subjected to alkaline hydrolysis.

For this purpose, 0·53 g. was heated with aqueous N-NaOH (20 ml.) for 1 hr. under reflux. The solution required 17·20 ml. of N-HCl for neutralization. The neutral solution was ex-

tracted with ether, giving 0·10 g. of oil, which was dissolved in 4 ml. of ethanol and treated with 2:4-dinitrophenylhydrazine in HCl (Brady & Elsmie, 1926). A red precipitate, 0·13 g., m.p. 244–246°, was obtained. The m.p. was raised to 251–252° on recrystallization from ethyl acetate—brilliant red plates. A further amount of the dinitrophenylhydrazone was prepared directly from the oily portion of the distillate without hydrolysis. This was shown by analysis and comparison (mixed m.p.) with an authentic sample of m.p. 252° to be the 2:4-dinitrophenylhydrazone of anisaldehyde. (Found: C, 53·27; H, 3·86; N, 17·5; MeO, 9·9. Calc. for $C_{14}H_{12}N_4O_5$: C, 53·16; H, 3·82; N, 17·71; MeO, 9·8%.)

The ether-extracted neutral solution from the hydrolysis on acidification yielded crystals (0·31 g.) of m.p. 176–181°, consisting of impure anisic acid. Thus Fraction I of the distillate consisted mainly of methyl anisate together with a small amount of anisaldehyde.

(*b*) *Fraction II.* 0·64 g., b.p. 140–175°/*c.* 1 mm. This was a yellow oil containing a little crystalline material. As it was acidic it was dissolved in ether and washed with saturated aqueous $NaHCO_3$.

(*c*) *Acids from Fraction II.* On acidification of the $NaHCO_3$ solution, acids (0·05 g.) were precipitated. They were subjected to fractional sublimation in high vacuum, and gave two main fractions: (1) 12 mg., m.p. 102–104°; (2) 17 mg., m.p. 166–176°, after softening above 120°. Sublimate (1) was still a mixture which could not be satisfactorily separated into its components. Sublimate (2) on recrystallization from benzene gave 8 mg. of an acid of m.p. 180–181°, which was shown by comparison (mixed m.p.) with an authentic sample to consist of anisic acid.

(*d*) *Neutral portion of Fraction II.* The ether solution which had been washed with $NaHCO_3$ contained 0·47 g. of oil which slowly developed crystals. A portion (0·19 g.) was subjected to alkaline hydrolysis by boiling with 5 ml. of N-KOH in methanol for 1 hr. The crystalline K salt which formed on cooling was dissolved by addition of water (20 ml.), and the liquid after extraction with ether to remove unsaponifiable matter was acidified with HCl. The crystals (needles, 0·09 g.) which separated had m.p. 160–165°, raised to 180–182° on recrystallization from benzene. This was again anisic acid (mixed m.p., no depression).

Fraction II therefore contained an ester of anisic acid—doubtless methyl anisate—and a small amount of free anisic acid together with traces of other unidentified acids.

Neither Fraction I nor Fraction II showed any marked uptake of bromine when dissolved in $CS_2$, which indicated the absence of appreciable amounts of cinnamic acid or derivatives.

## SUMMARY

The volatile metabolic products of the fungus *Trametes suaveolens* (Linn.) Fr. grown on aqueous malt extract were examined. Methyl anisate was identified as the major product; anisaldehyde was also detected, but is present in much smaller amount. These two products are probably the chief contributors to the odour of the fungus in culture. A little free anisic acid was also detected; this may arise by atmospheric oxidation of the anisaldehyde.

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# The Riboflavin Content of Food Served in Royal Air Force Messes

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(*Received* 31 *January* 1944)

As our knowledge of the role of riboflavin as an essential factor in human nutrition increases, the need for satisfactory estimation of the riboflavin content of food becomes more urgent. The microbiological method has been employed in many laboratories and has given reproducible results. However, if this method is to be accepted as reliable, it must give values in agreement with those obtained with other methods and in particular with those obtained with a reliable biological method. During the past two years food material prepared by a method described by Macrae, Henry & Kon (1943) has become available. Since this consisted of a complex mixture of foodstuffs it appeared to be suitable for comparative tests from which the reliability of the microbiological method of estimation of riboflavin in foodstuffs might be ascertained. Any substances which might possibly interfere with such tests were likely to be present in a mixture of this kind. This comparative study allowed a determination of the daily riboflavin intake of R.A.F. personnel.

## EXPERIMENTAL

### (*a*) *Materials*

The materials analyzed consisted of the total food available to five airmen or airwomen in their messes in this country at all meals during 7 days, or in the case of some of the airwomen's messes, the food available for breakfast, dinner and tea but not supper. The supper meal was collected separately because a large proportion of the personnel, mainly for social reasons, tended to take this meal away from their messes. The food was collected as served, stored at −20° and ground in the frozen state. The fine granules were then dried in a rapid current of air at 70°, and the dried material was again finely ground, well mixed and weighed before samples were taken for analysis. The final products contained approximately 5% of moisture. The average daily dry weight of the food served to one subject was calculated, and from this and the riboflavin content it was possible to determine the average daily intake of riboflavin by the personnel at the various R.A.F. Stations.

### (*b*) *Methods*

*Biological method.* The biological technique used in estimating riboflavin was that originally devised by El Sadr, Macrae & Work (1940) and modified for use with cereal products by Copping (1943). The test animals were 21-day-old black and white rats receiving a basal synthetic diet of pure maize starch 60 parts, casein ('ashless, extracted, riboflavin-free, G.L.', supplied by Glaxo Laboratories Ltd.) 20 parts, hardened arachis oil 12 parts, pure lard 3 parts and McCollum's normal salt mixture 5 parts, mixed with 100 parts of water and steamed for 3–5 hr. in order to dextrinize the starch and prevent the possible development of refection (Fridericia, Freudenthal, Gudjonsson, Johansen & Schoubye, 1927). The R.A.F. diets were given as supplements to the basal diet; the daily dose was 1–3 g. of the dried material, and this amount was readily consumed by the rats. From some preliminary tests a rough estimate of the riboflavin content of the diets was obtained and a suitable level selected for dosing which should yield satisfactory comparison with the standard daily doses of 6 and 12 $\mu$g. of pure riboflavin, which have been found to give a suitable slope in the curve of growth response. The results were calculated according to the method of Irwin (1944) as set out in a report of results obtained by the Vitamin A Sub-Committee.

*Microbiological method.* The microbiological method of riboflavin estimation was that of Snell & Strong (1939) as modified by Barton-Wright & Booth (1943). An amount of 5 g. of the dried R.A.F. diet was used in each estimation.

Experience has led to the introduction of modifications of the method of extraction of the riboflavin when dealing with these mixed diets. Both the airmen's and airwomen's diets had a high fat content, and the dried food was therefore defatted with light petroleum. The samples were then autoclaved at a pressure of 15 lb./sq. in. for 30 min. with about 50 ml. 0·25 N-HCl. The mixtures were allowed to cool and adjusted to pH 4·6 with NaOH. They were then vigorously shaken with ether in a separatory funnel to remove all interfering lipids released by the HCl. The lower aqueous layer, which contained the riboflavin, was run off and the ether layer washed several times with water, the washings being added to the aqueous layer. Finally, the extracts were adjusted to pH 6·8 and made up to 500 ml. with water. Clear solutions were thus obtained and all centrifuging and filtering obviated. The remainder of the determination was carried out as previously described, except that the addition of asparagine to the medium has been abandoned.

## RESULTS

The results given by the biological method were, with one or two exceptions, somewhat higher than those obtained by the microbiological method, but, on the whole, the agreement between the results with the two methods was good (Table 1). The microbiological method of determination would, therefore, appear to be reliable for the estimation

Table 1. *Riboflavin content of the diets served at various R.A.F. Stations, and the riboflavin intake of the personnel*

| R.A.F. Station | Riboflavin content of food (mg./100 g. dry matter) Method of assay: Rat growth | Riboflavin content of food (mg./100 g. dry matter) Method of assay: Microbiological | Average daily intake of: Food (g. of dry matter) | Average daily intake of: Riboflavin (mg.) Method of assay: Rat growth | Average daily intake of: Riboflavin (mg.) Method of assay: Microbiological | Energy (*e*) (cal.) |
|---|---|---|---|---|---|---|
| | | | (*a*) Airmen's messes | | | |
| 1 *a* | 0·26 | 0·25 | 568 | 1·5 | 1·4 | 2675 |
| 2 *a* | 0·31 | 0·29 | 635 | 1·9 | 1·8 | 3045 |
| 3 *a* | 0·48 | 0·43 | 625 | 3·0 | 2·7 | 3060 |
| 4 *a* | 0·31 | 0·29 | 592 | 1·8 | 1·7 | 2740 |
| | | | (*b*) Airwomen's messes | | | |
| 5 *a* | 0·45 | 0·37 | 532 | 2·4 | 2·0 | 2585 |
| 6 *e* | 0·23 | 0·27 | 459 | 1·1 | 1·2 } 1·6 | 2726 |
| 6 *s* | — | 0·38 | 106 | — | 0·4 } | |
| 7 *e* | 0·21 | 0·27 | 495 | 1·0 | 1·3 } 1·6 | 2622 |
| 7 *s* | — | 0·27 | 80 | — | 0·2 } | |
| 8 *e* | 0·38 | 0·37 | 510 | 1·9 | 1·9 } 2·2 | 2934 |
| 8 *s* | — | 0·38 | 89 | — | 0·3 } | |
| 9 *a* | 0·36 | 0·28 | 606 | 2·1 | 1·7 | — |

*a* indicates all meals included; *e* indicates all meals except supper; *s* indicates supper only.

of riboflavin in foodstuffs, having been found to give values similar to those obtained by the more costly and time-consuming rat-growth method.

The average daily intake of riboflavin per person was calculated from the average daily intake of dry food (Table 1). Intakes of less than 2 mg. of riboflavin for all except three of the messes were found with the biological method, and for all except two with the microbiological technique. The average daily intake per head was about 2·0 mg. for the airmen and 1·8 mg. for the airwomen. At the stations at which the supper meals were collected and dealt with separately, a rather large proportion of the daily energy and riboflavin intake appeared to be derived from the suppers. It is of interest that the airwomen's mess with the lowest riboflavin intake was one serving very much greater numbers than the other three airwomen's messes.

## DISCUSSION

If the standards for optimum requirement of vitamins, as set out by the Committee on Food and Nutrition of the National Research Council of the United States of America in 1941 (National Nutrition Conference), are taken as valid, the present values for riboflavin intake would appear to be suboptimal, since the daily requirement was there laid down as about 3 mg. for active men and 2·5 mg. for active women. The requirement for riboflavin has not, however, been finally established. Sebrell & Butler (1938), in a preliminary report of their findings on riboflavin deficiency in women, stated that cures were obtained with daily doses of 1–2 mg. in some cases, and with 0·025 mg./kg. body weight in others. These subjects, of course, received small amounts of riboflavin in their diets, but the total daily intake during the curing of the characteristic cheilosis could not have been more than 2 mg. in some instances. Since maintenance doses of any factor are usually considered to be less than curative ones, these experiments would indicate that the daily requirement of riboflavin is less than 2 mg. This is a lower estimate than the amount allowed in the National Research Council list, but it is confirmed by recent findings of Williams, Mason, Cusick & Wilder (1943), who attempted to induce riboflavin deficiency in human subjects by administration of a restricted diet, and then to restore the level of riboflavin in the tissues to normal again. From their studies they concluded that 0·5 mg. riboflavin/1000 cal. was an adequate allowance. In view of these results it would seem that the diets examined from the R.A.F. messes did supply an adequate daily intake of riboflavin, since the figures given in Table 1 show that at least 0·5 mg./1000 cal. was present, and in most diets there was a safe margin over this amount. Since the estimations were made on the food as served, all losses due to cooking and keeping of the hot food before consumption were covered.

Lyle, Macrae & Gardiner (1944) have observed no sign of deficiency of riboflavin in R.A.F. personnel in this country, which further supports the view that the normal adult requirements of riboflavin must be less than 3 mg. daily. It is true that in addition to the average of 1·8–2·0 mg. received daily in their messes, the personnel must have received some additional riboflavin from food consumed outside, but this could not have amounted to more than a small fraction of a milligram in most instances. Since beer is a surprisingly good source of riboflavin, containing about 0·5–1·7$\mu$g./g. (Barton-Wright, Moran & Sarson, 1943; Hopkins, 1943), beer drinkers would have a higher intake, but this would not affect the greater proportion of R.A.F. personnel as not more than 10% are regular beer drinkers.

## SUMMARY

1. Biological and microbiological methods are described for estimation of the riboflavin content of the food served in R.A.F. messes.

2. The values obtained by the two methods were in good agreement, though the results obtained by the biological method tended to be somewhat higher than those obtained by the microbiological method.

3. The daily intake of riboflavin by the personnel in their messes ranged from 1·5 to 2·6 mg. and averaged about 1·9 mg.

4. Since no sign of deficiency of riboflavin exists in the personnel receiving these diets, it is considered that the average riboflavin requirement of adults does not exceed 2 mg. daily.

Thanks are here recorded to Air Marshal Sir H. E. Whittingham, K.B.E., Director General Medical Services, Air Ministry, and Air Commodore T. McClurkin, Director of Hygiene, Air Ministry, for providing facilities for the carrying out of this work; to Sir Charles Martin, Roebuck House, Cambridge, for hospitality, and to the Medical Research Council for a personal grant to one of us (A. M. C.).

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# The Fluorimetric Estimation of 3:4-Benzpyrene in Whole Mice

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(*Received* 1 *February* 1944)

Previous methods for the quantitative estimation of carcinogenic hydrocarbons in animal tissues are based on spectrophotometric measurements of either the absorption spectrum (Chalmers, 1934; Jones, 1942; Lorenz & Shear, 1936; Lorenz & Shimkin, 1941; Lorenz & Stewart, 1940), or the fluorescence spectrum (Berenblum & Kendal, 1936; Berenblum & Schoental, 1942; Chalmers, 1938, 1940; Chalmers & Peacock, 1936; Hieger, 1936; Peacock, 1936). Although the greater simplicity of a fluorimetric estimation would result in a saving of time and money, its unspecificity, due to the presence of fluorescent impurities, was usually regarded as too great an obstacle. The present paper records methods of extraction and purification of 3:4-benzpyrene (henceforth called benzpyrene), as developed in this laboratory, and examines the possibilities and limitations of making allowance, in fluorimetric measurements, for the remaining impurities on a statistical basis.

In the course of investigations into the effect of lipoid solvents on the carcinogenic activity of benzpyrene it was decided to measure the rate of elimination of the hydrocarbon from the animal body after subcutaneous injection. As previously emphasized (Dickens & Weil-Malherbe, 1942), small doses of carcinogen should be employed in experiments of this kind, since otherwise any cocarcinogenic or anticarcinogenic properties of the solvent are liable to be smothered. Benzpyrene is particularly favourable, as it combines, relative to 20-methylcholanthrene and 1:2:5:6-dibenzanthracene, the highest fluorescence intensity with the lowest carcinogenic potency after subcutaneous injection (Bryan & Shimkin, 1943). A single subcutaneous injection of 0·3 mg. benzpyrene was found satisfactory in our experiments. The method has therefore been devised for a maximum amount of 0·3 mg. benzpyrene; it is, however, easily adapted to the estimation of larger amounts by modifications of the procedure of chromatographic purification, which will be discussed.

## SURVEY OF METHOD

The following three steps which are in principle, though not in detail, the essence of some previous methods, precede the actual estimation. (1) Lysis of the tissue in hot alcoholic NaOH. (2) Extraction of the hydrocarbon, together with non-saponifiable matter from the tissues, from alkaline 50% ethanol by light petroleum. (3) Purification of the petroleum extract by concentration and cooling and then by chromatographic adsorption and fractional elution (so-called 'liquid chromatography'). Benzpyrene is first adsorbed on a column of silica gel from light petroleum and eluted by light petroleum-benzene mixture (4 : 1). It is then readsorbed from this mixture on a column of alumina. After development by the same mixture it is eluted by benzene. (4) The concentration of benzpyrene is determined fluorimetrically.

## DESCRIPTION OF METHOD

### *Reagents*

*Purification of solvents*

*Ethanol*, 95% (v/v). Industrial methylated alcohol is mixed with 10N-$H_2SO_4$ (40 ml./l.) and rectified slowly through an efficient fractionating column (Clarke & Rahrs, 1923). This is followed by refluxing for 12 hr. over $AgNO_3$ (8 g./l.) + KOH (8 g./l.) and another rectification.

*Light petroleum.* The commercial product (b.p. 40–60°) is purified by the following successive extractions, each carried out with vigorous shaking for several minutes in a separator: nitrating mixture (125 ml. conc. $H_2SO_4$ + 100 ml. conc. $HNO_3$) (4 times), fuming $H_2SO_4$ (3 times), water and 25% NaOH. It is finally dried over $CaCl_2$ and rectified.

*Benzene.* Extraction of benzene (British Drug Houses Ltd., A.R.) with conc. $H_2SO_4$ (3–4 times), followed by washing with water and alkali, drying and rectification.

Ethanol may be recovered by fractional distillation, but before being used again it should be purified by the process described. The same applies to light petroleum recovered from light petroleum-benzene mixtures.

*Adsorbents*

*Silica gel* (British Drug Houses Ltd.). The gel is ground in a mortar and the 100–160-mesh fraction is screened out, care being taken to remove the dust, which would clog the adsorption column. The 60–100-mesh fraction is usable too and may be collected separately. Small batches of the adsorbent are dried at 110° for 24 hr., cooled *in vacuo* over $P_2O_5$ and stored in a glass-stoppered bottle, which is itself kept in a desiccator over $P_2O_5$.

*Aluminium oxide* (British Drug Houses Ltd., 'for chromatographic adsorption analysis') is dried at 300° for 3 hr. in small batches and cooled and stored like the silica gel.

Reproducible results can be obtained only if the adsorbents are standardized. The standardization must be repeated with every new sample, as products of different origin may show appreciable differences in adsorbing qualities. The method will be described later.

Adsorbents, alumina in particular, are extremely sensitive to atmospheric moisture, exposure to which entails a progressive reduction of adsorbing power. Once dried, a batch of adsorbent should therefore be protected from moisture and any unavoidable exposure reduced to a minimum.

*Lubricants*

The use of vaseline and other fluorescent lubricants on separator taps, etc., is to be strictly avoided. Suitable lubricants are lard, provided freshly greased taps are washed out with chloroform before use, and a mixture of graphite (previously extracted with hot benzene) and glycerol in such proportions that a thick, but smooth paste results. Contact with rubber is undesirable. The use of all-glass apparatus, without lubrication of joints, is recommended.

*Hydrolysis of tissues and extraction of benzpyrene*

The dead mouse is placed in a 300 ml. flask and 150 ml. 10% NaOH in ethanol are added. The latter is prepared by dissolving 1·2 parts of NaOH in 1 part of water and adding 11 parts of purified 95% ethanol (v/v). The contents of the flask are brought to boiling on a water-bath and refluxed for a further ½ hr. While still hot the solution is decanted from the skeleton into a 500 ml. flask. The flask and insoluble residues are rinsed three times with 5 ml. ethanol. After the addition of 150 ml. of water the cooled mixture is extracted in a separator with four successive lots of 50 ml. commercial light petroleum, b.p. 40–60°. (The purified solvent need not be used at this stage.) The combined extracts are dried over 5 g. $CaCl_2$ for about 2 hr. and then decanted into a 300 ml. distillation flask, followed by three washings with 10 ml. of light petroleum. If the extract or the washings or both are very cloudy, it is preferable to centrifuge them, though a moderate degree of turbidity is of no account. The petroleum extract is now evaporated to dryness, first under slightly reduced pressure and finally in the full vacuum of the water pump. The residue is taken up in purified light petroleum, added in lots of about 2 ml. at a time, and rinsed into a 10 ml. centrifuge tube until a volume of 6–8 ml. is reached. The extract is chilled in a freezing mixture for 20 min., being repeatedly stirred with a glass rod to assist crystallization. Finally, the solid matter is centrifuged off and washed three times with 0·5 ml. of chilled purified light petroleum.

*Chromatographic purification*

A series of thistle funnels is required with a bulb of 50 mm. diam. (approx.) and a neck which makes an airtight fit with a rubber bung. The stem of the funnel is 25 cm. long and has a 5–6 mm. bore. At a distance of 17 cm. from the bulb there is a constriction, on top of which is placed a small plug of glass-wool. The funnel is mounted on a small suction flask. The correct amount of adsorbent is measured out with a calibrated test tube, 10 × 75 mm., and poured into the funnel.

The filtrations are carried out under pressure, preferably from a $N_2$ cylinder, to avoid losses by evaporation. The pressure is applied to the top of the thistle funnels and is so regulated that the rate of filtration does not exceed 20–30 drops/min.

The clear petroleum solution is first passed through a column of 1·5 g. silica gel, followed by three washings of 2 ml. of purified light petroleum. The filtrate is rejected. 25 ml. light petroleum-benzene mixture 4 : 1 (both solvents purified) are passed through the column. The entire filtrate is then passed through a second column of 2 g. of alumina, followed by a further 20 ml. petroleum-benzene mixture, part of which is used to rinse out the flask previously used. The combined filtrates are rejected. Finally 15 ml. benzene (purified) are passed through the alumina column. The solution is now ready for fluorimetric estimation.

We have found it convenient to run four experiments simultaneously up to this stage.

*Fluorimetric estimation*

In this laboratory two fluorimeters are in use: a direct-reading apparatus built locally to our own specifications and the Spekker fluorimeter (Adam Hilger, Ltd.) which is a null-point instrument. The first-mentioned apparatus has been described in previous publications (Weil-Malherbe, 1943, 1944). Fig. 1 shows the tubes used with this apparatus. They contain 5 ml. solution of suitable concentration, i.e. a total of not more than 3$\mu$g. benzpyrene. A preliminary dilution of the final benzene eluate may be necessary. If the total amount of benzpyrene is less than 1–2$\mu$g. the benzene solution is concentrated *in vacuo* to less than 5 ml., made up to exactly 5 ml. and the total used for the estimation. A stream of $O_2$-free $N_2$, saturated with benzene, is passed through the solution for 2 min. before a reading is taken. A reference curve, which in this range of concentrations is practically linear, is established with standard solutions of known amounts of benzpyrene, treated in the same way. Every reading is corrected for a zero reading obtained with pure solvent.

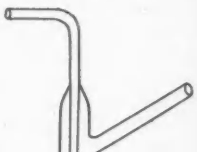

Fig. 1. Tube for use in the direct-reading fluorimeter.

For use with the Spekker fluorimeter special containers had to be made (Fig. 2), as the containers originally supplied

were not suitable for gassing; moreover, 20 ml. of solution were required to fill them, which caused a corresponding loss of sensitivity. The present container (Fig. 2, 2) (supplied by Tintometer, Ltd., Salisbury) is a fused glass cell with two parallel circular faces, of 35 mm. internal diam. and 10 mm. depth. Its capacity is 10 ml. The cell is provided with a gas inlet tube (3) and a two-bulb splash head (4).

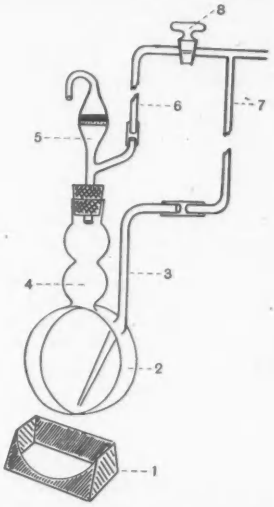


Fig. 2. Container with adaptor and gassing arrangement as used in the Spekker fluorimeter. For key to figures see text.

It is mounted on a brass adaptor without bottom (1) which fits exactly into the cell slide of the apparatus. Back-diffusion of $O_2$ is prevented by the mercury seal (5) made out of a fritted glass micro-filter tube. It is necessary to provide for equalization of pressure before taking a reading, as gas in the gas inlet tube causes total reflexion and prevents the ultra-violet light from penetrating into the solution beyond. If the gas inlet tube is filled with solution, fluorescence is evenly distributed within the range of the beam of ultra-violet radiation. $O_2$-free $N_2$, saturated with benzene, is bubbled through the solution for an appropriate time (3–5 min.) with tap 8 closed. Before a reading is taken the tap is opened, which causes the solution to fill the gas inlet tube while the gas stream escapes through the short-cut. The box which encloses the photoelectric cell and the container of the fluorescent solution must be enlarged to accommodate the modified vessel. This is done by unscrewing the lid with its hinges and fitting a brass extension of 95 mm. height to the back wall. A similar extension of the same height is fitted to the lower end of the three sides of the lid, which is then remounted on top of the extended back wall. The two gas-leading tubes (6 and 7) enter the box through holes in the extension of the back wall.

Solutions of quinine sulphate in N-$H_2SO_4$ are used as standards of fluorescence, a solution containing 3$\mu$g./ml., if 10 ml. of the unknown solution contain 0·5–2$\mu$g. benzpyrene, and a solution containing 10$\mu$g./ml., if 10 ml. of the unknown solution contain 2–10$\mu$g. benzpyrene. Stronger solutions of benzpyrene should be suitably diluted. The vessel used for the standard solution is an ordinary rectangular glass cell, and the measurement is carried out without preliminary gassing. Calibration curves are established for both concentrations of standard solution with known amounts of benzpyrene and the results plotted on semi-logarithmic graph paper. The resulting curves are linear.

The colour filters between fluorescing solution and photocell are the same as those used with the direct reading apparatus (Weil-Malherbe, 1944), viz. Wratten filters nos. 35 + 43, with a transmission maximum at about 400 m$\mu$.

### *Calculation of results*

Two corrections are applied to the final result, after allowance has been made for the sample used: first an amount of 0·5$\mu$g. is subtracted to allow for the blank; the remainder is then multiplied by a factor 1·145 to allow for a fairly constant loss, which amounts to 13 % on the average.

## DISCUSSION OF METHOD

### *Extraction*

Berenblum & Kendal (1936) and Berenblum & Schoental (1942) claim that dibenzanthracene and benzpyrene cannot be extracted quantitatively from an *alkaline* ethanol solution; and that quantitative extraction is possible only after acidification. No such difficulties, for which indeed no theoretical basis can be seen, were observed under our conditions, which differ from those of Berenblum *et al.* with respect to (1) dilution of the alcoholic solution with an equal volume of water before extraction and (2) the choice of light petroleum instead of benzene as solvent. It seems not unlikely that the effect ascribed by Berenblum *et al.* to acidification is in fact due to dilution with water, or is a salting-out effect.

### *Chromatographic purification*

The method adopted is based on a detailed quantitative study of chromatography (Weil-Malherbe, 1943) aimed at finding empirical rules which would allow the prediction of the effect of varying such factors as concentration or quantity of the substance to be adsorbed, quantity or quality of the adsorbent, quality of the solvent, etc. It was shown that for the analysis of a simple chromatographic system the estimation of two parameters is sufficient, Freundlich's adsorption isotherm coefficient and the threshold volume $V_t$. The latter is defined as the volume of filtrate collected up to the beginning of elution, provided only one solvent is used for adsorption, development and elution. Whereas the adsorption isotherm is a constant for any given system, $V_t$ is a variable depending on the variation of the factors mentioned and related to them by simple empirical expressions. With their aid it is possible to devise a procedure of such a nature that it is capable of coping with any specified range of concentrations. In the application of this principle to the purification of benzpyrene the upper limit of the concentration range was chosen at 300$\mu$g., as this was the dose injected. The lower limit was taken at 0·5$\mu$g., as the blank made the fluorimetric estimation of smaller amounts too uncertain. In the procedure finally adopted a wider margin of safety was chosen than that originally proposed (Weil-Malherbe, 1943). It was found that this could be done without increasing the blank in any way.

*Adaptation of the procedure to different ranges of benzpyrene concentration*

The exponential coefficient of Freundlich's adsorption isotherm for the system silica gel, benzpyrene, light petroleum, benzene, was found to be very nearly 1. This means that the amount adsorbed on a given amount of adsorbent is almost proportional to the concentration of the initial solution and $V_t$ is almost independent of the concentration of the initial solution. It will therefore not be necessary to modify either the quantity of silica gel in the column or the volume of solvent used for elution, even though the range of concentration be considerably extended in either or both directions, especially as a wide margin of safety has already been allowed. The alumina system, however, is less elastic. Here $V_t$ changes inversely with the concentration of the initial solution: increasing the latter means either reducing the volume of solvent used for development or increasing the amount of adsorbent. The empirical rules governing $V_t$ give an idea of the magnitude of the effect to be expected. Thus, using the same constants as were employed in the numerical example on p. 310 of the paper by Weil-Malherbe (1943), we find $V_t = 56$ ml. for the case in which we pour a solution of 300 $\mu$g. benzpyrene, dissolved in 25 ml. light petroleum-benzene mixture, on to a column of 2 g. of alumina. With an increase of the quantity of benzpyrene to 1000 $\mu$g., $V_t$ is reduced to 43 ml. On the other hand, if we increase the amount of adsorbent to 2·5 g., $V_t$ for 1000 $\mu$g. is the same as $V_t$ for 300 $\mu$g. and a column of 2 g., viz. 56 ml. In this case the volume of solvent used for development need not be reduced, but account must be taken of the fact that $V_t$ for 0·5 $\mu$g. benzpyrene is increased from 2·8 ml. benzene to 3·9 ml. benzene. So, if it is desired to maintain the lower range of concentrations at the same level as before, the volume of benzene used for the final elution must be increased in the same proportion, i.e. from 15 to 20 ml.

These considerations may seem superfluous, since it is often possible to observe the progress of development and elution by the fluorescence of benzpyrene in filtered ultra-violet light. Might it not be preferable simply to collect the fluorescent fraction of the filtrate as determined by direct observation in every individual case, rather than to maintain a fixed routine capable of dealing with a whole range of concentrations? There are three reasons in favour of the latter course. (1) In the evaluation of the fluorimetric measurement a correction is applied for the blank. It is therefore essential that the blanks of different experiments should be strictly comparable. Yet there is no guarantee that modifications of the procedure of purification will leave the blank unaltered. (2) With low concentrations of benzpyrene ($<10\mu$g.) fluorescence becomes so weak, especially in solvents containing light petroleum, that direct observation is no longer possible. Even with higher concentrations it is very difficult to decide when the elution is complete. (3) A routine procedure has the advantage of making the analyst independent of the ultra-violet lamp. This does not mean that the procedure should not be controlled from time to time by direct observation in ultra-violet light.

*Standardization of adsorbents*

The procedure of chromatographic purification described has been worked out for adsorbents with strictly defined adsorbing qualities. Adsorbents of unknown quality have to be standardized before they are used in the estimation. This is done by determining the value of $k$ for each adsorbent, $k$ being defined by the equation

$$k = \frac{V_t}{s^{1/\alpha}},$$

where $V_t$ is the threshold volume in ml., $s$ the amount of adsorbent in g. and $\alpha$ the exponential coefficient of Freundlich's adsorption isotherm. The equation holds only if the volume and concentration of the initial solution are kept constant. For the purpose of estimating $k$ it is convenient to use a solution of 300 $\mu$g. benzpyrene in 1 ml., as initial solution. Strictly speaking, $\alpha$ should be estimated separately for every system investigated, but it will probably introduce no serious error if the values found in this laboratory are assumed, viz. $\alpha = 0{\cdot}67$ for the alumina system and $\alpha = 0{\cdot}96$ for the silica gel system. For testing alumina a mixture of light petroleum-benzene 4 : 1 is used as solvent and for silica gel 3% benzene in light petroleum (purified solvents). The experiment is done in triplicate with columns of 2 or 3 g. adsorbent. After addition of the initial solution, pure solvent is poured on, and the progress of development followed in ultra-violet light. It is not necessary to determine $V_t$ accurately by extrapolation from the data of a complete elution curve (cf. Weil-Malherbe, 1943) for this purpose. The filtration is stopped when the first fluorescent drop leaves the column, the volume of the filtrate is measured and the mean value is used as $V_t$ for the calculation of $k$. Alumina is satisfactory if its $k$-value is found to lie between 15 and 18, silica gel if its $k$-value is between 19 and 21. If the adsorbent tested has a $k$-value which differs considerably from these figures, the amounts used must be modified according to the equation

$$s_2 = s_1 (k_1/k_2)^{\alpha},$$

where $s_1$ is the weight and $k_1$ the constant of the standard adsorbent and $s_2$ the weight and $k_2$ the constant of the adsorbent to be used.

*Fluorimetric measurement*

Theoretically, a null-point instrument, such as the Spekker fluorimeter, should give more accurate results, as any fluctuations in output of ultra-violet light are automatically compensated. In practice, however, the direct-reading instrument was found just as satisfactory and in many respects even preferable. Light fluctuations caused no serious trouble; moreover, they can be guarded against by use of a voltage stabilizer and by frequent reference to the standard solution. The advantages are: simple and rapid manipulation without the necessity of making meticulous adjustments; readings directly proportional to fluorescence intensity; more convenient arrangement for gassing and for insertion and change of containers; superior sensitivity owing to higher energy output of illuminating lamp, larger photo-cell and smaller volume of test solution.

The choice of the colour filter with transmission maximum at 400 m$\mu$. was actuated by the finding that it reduced the blank, expressed in units of benzpyrene, in a higher degree than did filters with transmission maximum at longer wave-lengths; specificity is thereby increased. A similar gain of specificity is achieved by carrying out the measurement in an atmosphere of pure $N_2$. This is due to the fact that the blank is less subject to the quenching effect of $O_2$ than benzpyrene. Other advantages of eliminating $O_2$ are: a greater sensitivity owing to the increased fluorescence intensity, and an almost complete independence from solvent effects, i.e. variations of fluorescence intensity according to the solvent used (Weil-Malherbe & Weiss, 1942).

## RESULTS

### *The blank value*

Crude petroleum extracts of benzpyrene-free mice, after concentration to about 6–8 ml., are usually faintly yellow and show an intense white or yellow fluorescence. After chromatographic purification the fluorescence is a pure blue. A faint blue fluorescence is even shown by solutions from experiments without tissue. This latter value is referred to as 'reagent blank', while the value found with benzpyrene-free mice is called the 'blank'. The blank is therefore of composite nature, as both reagents and mouse contribute to it. As the accuracy and sensitivity of the method are limited by the blank, its magnitude and distribution have been studied in numerous analyses. The blank has throughout been measured in units of benzpyrene.

*Reagent blank*

The reagent blank was usually found to lie between 0·10 and 0·15 $\mu$g. If only that part of the analysis is carried out which starts with the chromatographic purification, the reagent blank amounts to about 0·08 $\mu$g. The origin of the other more variable part of the reagent blank could not be traced with certainty to any particular reagent or phase of analysis; an atmospheric impurity is not responsible, as shown by experiments in which air was filtered through cotton-wool during evaporations under reduced pressure, and by other experiments in which unfiltered air was sucked through benzene for about 8 hr.

*The mean value of the blank*

The mean blank of 54 analyses was found to be $0{\cdot}50 \pm 0{\cdot}01$* $\mu$g., the standard deviation of a single observation (S.D.) being $0{\cdot}117 \pm 0{\cdot}0076$.* To give a significant, if only qualitative, indication of the presence of benzpyrene a reading should not be less than 0·85 $\mu$g. (i.e. mean + 3 S.D.). In fact, the highest value of the blank observed was 0·74 $\mu$g. and the lowest 0·27 $\mu$g., both being observed only once. Of the 54 animals 32 were males and 22 females; the mean blank value for the males was 0·49 $\mu$g. (S.D. 0·107) and that for the females 0·51 $\mu$g. (S.D. 0·119). The difference does not exceed the probable error of the mean for both groups combined and is therefore not significant. If the animals were grouped according to their weights regardless of sex, a group of 24 animals weighing less than 30·0 g. (av. wt. 27·2 g.) had a mean blank of 0·46 $\mu$g. (S.D. 0·114), whereas a group of 27 animals weighing more than 30·0 g. (av. wt. 36·9 g.) had one of 0·53 $\mu$g. (S.D. 0·127). The difference between the means is significant ($P = 0{\cdot}03$). Thus it seems that the blank increases with the weight of the mouse.

* Probable error.

*Chemical nature of the blank*

Various experiments, which were carried out in an attempt to reduce the blank, also permit some—mainly negative—conclusions as to the chemical nature of the material responsible for it.

The possibility that the blank might result from autoxidation during the phase of alkaline hydrolysis was ruled out by an experiment in which the hydrolysis was carried out in an atmosphere of pure $N_2$. No difference in the blank value resulted.

In order to test the homogeneity of the material responsible for the blank, residues from over 50 blank experiments were pooled and evaporated to dryness *in vacuo*. They were taken up in 10 ml. light petroleum and passed through a column of 2 g. of silica gel. A single band with grey-blue fluorescence formed, and was washed out by 25 ml. light petroleum-benzene mixture 4 : 1. The filtrate was now passed through a column of 2 g. of alumina. On development with more of this mixture the single band separated into two, which were at no stage completely parted, but showed a continuous transition. The lower band had a dull grey-blue fluorescence and the upper a light blue fluorescence. The eluates of the two bands were collected separately and the following results were obtained in experiments with portions of each fraction: (1) neither fraction formed an alkali-soluble semiester with succinic or phthalic anhydride, which indicated the absence of alcoholic hydroxyl groups; (2) neither fraction reacted with trimethylammonium acetohydrazide chloride, which indicated the absence of carbonyl groups; (3) neither fraction reacted with digitonin, which indicated the absence of sterols of the $\beta$-type; (4) after extraction of 5 ml. samples in light petroleum by 2, 1, 1 ml. portions of conc. $H_2SO_4$ the fluorescence of both fractions was reduced by more than 50%; (5) the fluorescence of neither fraction is appreciably affected by shaking with oxidizing agents in alkaline medium (e.g. $Na_2O_2$, alkaline $KMnO_4$). It is diminished to some extent by shaking with oxidizing agents in acid medium. In this respect the behaviour is similar to that of benzpyrene, though the latter is somewhat more susceptible to oxidizing agents in acid medium. The difference is, however, not sufficiently great to allow a differential estimation.

*Recovery of added benzpyrene*

The fluorescence intensities of the blank and benzpyrene are strictly additive, as ascertained in experiments where a known amount of benzpyrene was added to a previously measured blank solution. The recovery in the method as a whole was tested by adding known amounts of benzpyrene, varying from 3 to 300 $\mu$g., dissolved in 1 ml. of light petroleum, to the mixture of mouse and alcoholic

Table 1. *Recovery of benzpyrene added to whole mouse*

| Benzpyrene added (μg.) | No. of analyses | Mean recovery of benzpyrene (μg.) | (%) | Standard deviation (%) |
|---|---|---|---|---|
| 300 | 20 | 263·7 | 87·9 | 2·48 |
| 100 | 4 | 85·3 | 85·3 | 0·65 |
| 30 | 4 | 26·5 | 88·5 | 2·68 |
| 10 | 4 | 8·62 | 86·2 | 4·86 |
| 3 | 4 | 2·58 | 86·0 | 3·14 |
| Total | 36 | Averages | 87·36 ± 0·32 | 2·82 ± 0·22* |

* Probable error.

NaOH before hydrolysis. Table 1 shows the results obtained. No correction for the blank was made in the analyses with 300 μg., where such a correction would be clearly insignificant; but when smaller amounts of benzpyrene were added, 0·5 μg. was subtracted from the recovered quantity to allow for the blank. The recovery falls short of the theoretical figure by about 13 %. But this error is fairly constant within the range studied and can therefore be corrected by multiplying the result with a factor of 1·145. This procedure will introduce an error of 6·3 % or more in 5 % of cases or less.

*Accuracy and sensitivity*

When the results of the 36 analyses are corrected by the factor 1·145, the errors shown in Table 2, expressed as standard deviations of the analytical results from the theoretical values, are obtained. The accuracy of the method does not diminish appreciably with diminishing quantities of benzpyrene within the range of 3–300 μg.

Table 2. *Accuracy of benzpyrene estimations*

| Benzpyrene added (μg.) | No. of analyses | Standard error (after correction for blank and recovery) (%) |
|---|---|---|
| 300 | 20 | ± 2·8 |
| 100 | 4 | ± 2·3 |
| 30 | 4 | ± 3·0 |
| 10 | 4 | ± 5·0 |
| 3 | 4 | ± 3·4 |
| 0·5 | 10 | ± 25·5 |

The limit of sensitivity of the method is determined by the blank. When the difference between the fluorescence intensities of benzpyrene and blank becomes small, the errors due to deviations of the blank from the average value used for correction will increase. With quantities of benzpyrene > 3 μg. this error will be insignificant compared with the other errors of the method. But with smaller amounts it assumes larger proportions and the other errors become correspondingly less important. Thus, if we take no account of the variations of recovery, the standard deviation of the mean blank (0·117 μg.) shows that in the estimation of 1 μg. benzpyrene a standard error of 11·7 % is to be expected. For 0·5 μg. this error will be doubled. The standard error which was actually observed in 10 analyses is very close to the expected figure (Table 2).

## DISCUSSION OF RESULTS

The accuracy of the method is satisfactory if the amount of benzpyrene is not less than 1 μg. As this is only 0·3–0·6 % of the marginal dose necessary for tumour production, the sensitivity of the method is sufficient for most purposes. If it is desired to estimate smaller amounts, the spectrophotometric method must be employed which, according to Berenblum & Schoental (1942), has a lower limit of sensitivity of 0·05 μg. benzpyrene/mouse.

If a standard of accuracy is admissible where a standard error of 25 % is no serious objection, even results of 0·5 μg. by the present method may still be valuable. Results of 0·3–0·5 μg. benzpyrene should be regarded as qualitative only.

## SUMMARY

1. A method is described for the fluorimetric estimation of 3:4-benzpyrene in mice. The range of the method extends from 0·5 to 300 μg., but it is easily adaptable to the estimation of larger quantities.

2. The principal steps in the method are: lysis of the tissue in hot alcoholic NaOH, extraction with light petroleum, purification by chromatographic adsorption and fractional elution, and finally photoelectric measurement of the fluorescence intensity.

3. The evaluation of results requires the application of corrections for a blank and for incomplete recovery of added benzpyrene.

4. The limit of sensitivity is determined by the blank, i.e. the fluorescence intensity of extracts from benzpyrene-free mice. The average value of 54 experiments was found to be 0·50 μg. in terms of benzpyrene. The blank is the same in both sexes, but increases somewhat with the weight of the animal.

5. The mean recovery of 36 experiments ranging from 3 to 300 μg. benzpyrene was found to be 87·36 %.

6. The standard error of the same recovery experiments, after correction for blank and losses, varied between 2·3 and 5 %. It rose to 25·5 % for 0·5 μg., as compared with a standard error of 23·4 % to be expected on the basis of the standard deviation of the blank.

The cost of the Hilger Spekker Absorptiometer and Fluorimeter used in these experiments was largely defrayed by an allocation made to this Council by the Imperial Cancer Research Fund from the bequest of the late Mrs M. I. Gibson of Northumberland.

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# A Note on the Fluorimetric Estimation of 3:4-Benzpyrene by the Method of Miller and Baumann

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After completion of the preceding paper (Weil-Malherbe, 1944*b*) a communication by Miller & Baumann (1943) on the fluorimetric estimation of carcinogenic hydrocarbons came to hand. These authors describe an ingenious method of 'two-condition fluorimetry' in which use is made of the different susceptibility to the quenching effect of $O_2$ of hydrocarbons on one hand and tissue material on the other (cf. Weil-Malherbe, 1944*a*). If the quenching ratios of both are known, the composition of a mixture can be deduced from the increment of fluorescence obtained on changing from an oxygenated to an oxygen-free medium.

The method has been tested in the following experiment with amounts of 0·2, 0·3 and 0·5 $\mu$g. benzpyrene added to three previously purified mouse extracts. Benzene was used as solvent and the measurements carried out first in pure $N_2$ and then in pure $O_2$. The fluorescence ratios (cf. Miller & Baumann, 1943) were: $N = 8{\cdot}8$, $n = 3{\cdot}8$, $K$ ($\mu$g. benzpyrene/unit of fluorescence in $O_2$) = 0·032.

| Benzpyrene added ($\mu$g.) | Reading in $N_2$ | Reading in $O_2$ | Benzpyrene recovered ($\mu$g.) |
|---|---|---|---|
| 0·2 | 227 | 51 | 0·21 |
| 0·3 | 218 | 45 | 0·30 |
| 0·5 | 272 | 52 | 0·48 |

These results are very satisfactory and there is no doubt that the method of Miller & Baumann will extend the range of sensitivity of the fluorimetric method considerably.

One point of Miller & Baumann's method could not be confirmed. They use crude, unpurified extracts directly for the fluorimetric analysis. These crude extracts are, at any rate as far as whole mice are concerned, entirely unsuitable for fluorimetry for the following reasons: (1) They very often contain finely suspended matter which cannot be removed by centrifugation or which reappears on standing after centrifugation. This is especially true for unchilled extracts. (2) They usually contain yellow pigments of animal origin, and other material, which exert a filter effect by absorbing either the primary or the secondary light. Their fluorescence is white or yellow with hardly any blue component; thus with a violet or blue colour filter the paradoxical result is obtained that they cause a smaller reading than the purified extract. Addition of benzpyrene to these crude extracts does not result in a corresponding increase of fluorescence, as shown by the following experiment:

| Extract | Dilution* | Colour | Reading before and after the addition of 1 $\mu$g. benzpyrene: Before | After |
|---|---|---|---|---|
| None | — | — | — | 118 |
| 1 | 1 in 20 | Colourless | 0 | 71 |
| 2 | 1 in 2 | Colourless | 13 | 61 |
| 3 | 1 in 2 | Yellow | 0 | 4 |

* Compared with the concentration used for fluorimetry on purified extracts.

It follows (1) that yellow extracts may blot out the fluorescence of benzpyrene completely, (2) that even colourless extracts may reduce the fluorescence appreciably, and (3) that they may do so even after twentyfold dilution. It is unlikely that the technique of hydrolysis and extraction used by Miller & Baumann for large amounts of tissue, which is practically identical with that described in the preceding paper, would have yielded less impure extracts. It seems, therefore, that Miller & Baumann's method, if applied to whole mice, does not dispose of the necessity of purifying the extracts.

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# Chemistry of Tissues

## 1. CHONDROITIN FROM CARTILAGE

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Mucoproteins, mucoids, and glycoproteins belong to the class of compound constituted of protein and carbohydrate in combination, a recent new classification of which has been given by one of us (Stacey, 1943). The discovery that compounds of this type give rise on hydrolysis to sugars was made by Eichwald (1865), and since that time much scattered and fragmentary evidence concerning their constitution and behaviour has been reported.

Mucoproteins were roughly distinguished from other proteins by the fact that they formed viscous solutions in water from which they could readily be precipitated in the cold by the addition of acetic acid. Eichwald studied the mucins of *Helix pomatia*, lymph, thymus, and salivary glands, and found that protein-carbohydrate complexes could be obtained from each of these sources, findings which were corroborated by later workers. Owing to the difficulty at that time of distinguishing readily between individual sugars, Eichwald wrongly concluded that the reducing substance which was produced by the hydrolysis of mucoproteins was glucose. Subsequent investigations by other workers, including Obolenski (1871) and Wolfenden (1884), showed, however, that this substance differed from glucose in that it contained nitrogen; while Müller (1901), by isolating from the mucin of bronchial mucus both a benzoyl derivative and the hydrochloride of chitosamine, showed that the reducing substance was an amino sugar. Landwehr (1882) considered that the mucin of *H. pomatia* contained not a simple sugar, but a N-free polysaccharide of the same type as occurs in the plant gums. While N-free carbohydrates do exist in some mucoproteins, N usually is present and is in part present as amino-sugar residues. Speculation arose early as to whether there was chemical combination between the protein and polysaccharide in mucoproteins, or whether, as Landwehr considered to be the case, they were present together in the form of a mechanical mixture. It is now generally accepted that they exist in chemical union, possibly in the form of a salt-like combination.

As a result of numerous investigations it was recognized that two fairly well-defined polysaccharides, namely, chondroitin sulphuric acid (herein termed chondroitin sulphate) and mucoitin sulphuric acid, were constituents of many mucoproteins, and these, especially the former, have been the subject of most of the detailed work which has been carried out in this field. Many mucopolysaccharides too (Meyer, 1938; Stacey, 1943) have been isolated from a correspondingly large number of sources, but there is little agreement among workers as to their individuality and constitution.

Levene (1925) devoted considerable care to the isolation of many mucoproteins and mucopolysaccharides, but no structural investigations were satisfactorily completed. Indeed, the only polysaccharide in this class which has been investigated in detail is that obtained from ovomucoid (Stacey & Woolley, 1940, 1942).

The group of acid mucopolysaccharides which contain organic sulphate residues includes heparin, the blood anticoagulant factor, as well as chondroitin sulphate and mucoitin sulphate from gastric mucin and possibly types of hyaluronic acid. From the preliminary examination of these carbohydrates all three appear to be similar in composition, consisting of *N*-acetyl amino sugars combined with glucuronic acid residues and esterified with $H_2SO_4$. In chondroitin sulphate the amino sugar is chondrosamine (2-aminogalactose or 2-aminotalose) (Levene, 1925), although in mucoitin sulphate (Levene, 1925) and in heparin (Jorpes & Bergström, 1937) the sugar is glucosamine. Chondroitin sulphate occurs in connective tissue, but its chief sources are cartilage from nasal septa, tendons, aorta, trachea, and sclera. It is perhaps the most readily accessible sulphur-containing polysaccharide. Mucoitin sulphate is found in cornea and gastric mucin, while heparin occurs in many organs, the best source yet discovered being the liver. Chondroitin sulphate, in common with other sulphated polysaccharides, exhibits a weak anti-coagulant action. Since chondroitin sulphate is probably related structurally to heparin, an investigation of its constitution may give information of use in the study both of heparin and of other carbohydrate sulphuric esters.

Chondroitin sulphate was first obtained by Fischer & Boedeker (1861), and it was not until 1884 that a reasonably pure sample was prepared by Krukenberg (1884). Methods of preparation and, consequently, the purity of the specimens obtained have not greatly improved since that time. The first constitutional studies were made by Schmiedeberg (1891) and, although his conclusions are not always satisfactorily founded upon experimental evidence, he at least is responsible for the basis of the present-day conception of the structure of chondroitin sulphate.

Levene's (1925) conception of chondroitin sulphate is that of a tetrasaccharide compound of glucuronic acid (2 units) and chondrosamine (2 units) conjugated with $H_2SO_4$ residues. On hydrolysis with HCl it gives a disaccharide 'chondrosin' which is formed by the union of one residue to chondrosamine with one of glucuronic acid, the points of attachment between which are assumed.

K. Meyer and his colleagues have conducted many careful analytical and preparative studies on sulphated polysaccharides, including chondroitin sulphate. Thus Meyer, Palmer & Smyth (1937) and Meyer (1938) studied the complexes which chondroitin sulphate forms with proteins. They concluded that the complexes were salts involving the —COOH and $—SO_3H$ groups in chondroitin sulphate and $—NH_2$ groups in the proteins. The interesting suggestion

was made that hyaline cartilage is a salt of chondroitin sulphate with a protein similar to gelatin, and the resemblance of artificial edestin-chondroitin sulphate fibres to natural elastic fibres of connective tissue was noted. Meyer & Smyth (1937) described a method of preparing chondroitin sulphate from cartilage, which involved an extraction with 10% $CaCl_2$ solution followed by deproteinization. The products differed slightly from the chondroitin sulphates described above in having N, 2·95%; S, 5·8%; and $[\alpha]_D$ −17°. These methods appear to cause very little degradation of the molecule, particularly with respect to hydrolysis of sulphate residues. No structural details were presented by the authors.

The investigations described below were begun some years ago and were unavoidably interrupted when only the preliminary stages had been carried out. However, sufficient evidence has been accumulated to show that chondroitin possesses a complicated branched chain structure and that Levene's (1925) formula for chondroitin sulphate is no longer tenable.

## EXPERIMENTAL

*Preparation of chondroitin sulphate.* (1) *From nasal septa.* The polysaccharide was prepared from the nasal septa of cattle by a modification of the method of Levene (1925). The septa were freed from flesh, bone, fat and other matter, finely chopped and kept for 2–3 days in contact with 2% aqueous KOH. The resulting viscid liquid was filtered through cloth and neutralized with glacial acetic acid. $BaCO_3$ (100 g./l.) was then added and the mixture heated on a boiling water-bath for 8–10 hr. The filtered solution was concentrated and the crude polysaccharide, precipitated by adding glacial acetic acid (8 vol.), was filtered and washed with ethanol. It was purified by dissolving in dilute KOH (pH about 8·0); the undissolved matter was removed (centrifuge) and the product in the supernatant liquid precipitated with ethanol (3 vol.). The polysaccharide, in the form of the crude K-salt, was separated (centrifuge), washed with absolute ethanol and ether, and dried in a vacuum. It was a white amorphous powder having $[\alpha]_D^{20°}$ −26·2°; ash (K), 19·2% (cf. Table 1).

(2) *From bovine trachea, etc.* The tracheae were freed from flesh as above, cut into small pieces and dissolved rapidly in N-NaOH at 70°. The resulting solution was neutralized with acetic acid and heated with $BaCO_3$ (100 g./l.) for 6 hr. The solid material was then separated and washed with water (centrifuge) and the supernatant liquid treated with a large excess of glacial acetic acid to precipitate the polysaccharide, which was separated (centrifuge) and further purified, either by Sevag's (1934) method or by repeated reprecipitation from aqueous solution by means of ethanol. It was washed with absolute ethanol and ether and then dried.

No other element except Na was detected in the ash from the trachea polysaccharide, and on this assumption the equivalent weight of this sample was 550. It is considered, however, that, since the products contain both free —COOH groups and —$SO_3H$ groups, equivalent weights obtained by direct titration are of little value.

*Reprecipitation of the polysaccharide from acid solution.* Attempts were made to reduce the ash content of the polysaccharide by reprecipitation by ethanol from dilute HCl. In the case of the septa polysaccharide after a single reprecipitation, the ash content was 11·4% (weighed as sulphate) and the rotation $[\alpha]_D$ −23° in water. This polysaccharide was acid in solution and its equivalent weight determined by titration with 0·01 N-NaOH was 538. Further precipitation from dilute HCl resulted in a further reduction in the ash content, but after six precipitations the polysaccharide was reducing to Fehling's solution, and had an ash content of 6·3% (as sulphate). The ash could not be removed by further precipitation. Other samples of polysaccharide gave comparable results. The reducing nature of the product may indicate that in the natural chondroitin sulphate some reducing groups are substituted by sulphate groups.

*Autolysis of chondroitin sulphate.* The polysaccharide obtained by a single reprecipitation from acid solution (see above) was non-reducing, but, on being heated in aqueous solution on a water-bath for 5 hr., became strongly reducing, suggesting that autolysis had taken place.

*Preparation of degraded chondroitin.* The polysaccharide was dissolved in 0·1 N-$H_2SO_4$ (20 g./l.) and heated on a water-bath for 30 hr.; $[\alpha]_D^{20°}$ −26° → −5° (*c*, 0·2) (constant value). The acid was then neutralized with $BaCO_3$, the filtered solution concentrated under diminished pressure, and the degraded polysaccharide precipitated with ethanol (5 vol.). It was isolated in the usual way. The supernatant fluid contained a small amount of syrupy material (4%) which gave a positive test for amino sugar, and contained sulphate residues. This syrup probably was chondrosamine sulphate.

'Degraded chondroitin', an oligosaccharide, was non-reducing to Fehling's solution and contained traces only of sulphate residues. The Ba salt had ash ($BaSO_4$) 25·0% and an equivalent weight of 465, which corresponds to that of a trisaccharide.

*Preparation of acetylated degraded chondroitin.* The acetylation was carried out by allowing a suspension of the degraded polysaccharide, in pyridine containing acetic anhydride, to shake for 48 hr. at room temperature. The reaction mixture was centrifuged and the supernatant liquid concentrated to a syrup. The acetylated derivative of the degraded polysaccharide was precipitated from a chloroform solution of this syrup by the addition of excess light petroleum. The solid layer, which was separated by centrifuging, was reacetylated as before, this process being repeated until the whole of the degraded polysaccharide had been acetylated. The product was a yellow powder with OAc, 38%.

*Methylation of acetylated degraded chondroitin.* The acetyl derivative prepared as above (10 g.) was dissolved in water

Table 1. *Properties of different specimens of chondroitin sulphate*

| Source | Ash, as sulphate (%) | Inorganic elements | $[\alpha]_D^{17°}$ | N (%) | S (%) | OAc (%) |
|---|---|---|---|---|---|---|
| Bovine nasal septa | 19·2 | K, Ba | −26·0° | 3·0 | 2·3 | 8·5 |
| Bovine trachea | 13·05 | Na | −25·0° | 4·3 | 2·5 | 8·6 |
| Human trachea | 15·0 | Na | −25·0° | 4·0 | 2·5 | 8·4 |
| Human cartilage | 34·6 | Na, Ba | −19·0° | 4·9 | 4·1 | 7·4 |

(100 ml.), and carbon tetrachloride (300 ml.) added. The mixture was stirred with methyl sulphate (80 ml.), and 30% aqueous NaOH solution (170 ml.) was run in over a period of 1 hr. Methyl sulphate (75 ml.) and alkali (158 ml.) were then added simultaneously over a period of 2 hr., and the mixture stirred at 35° for 2 hr. Some of the $Na_2SO_4$ crystallized and was filtered off. The filtrate was concentrated and the product remethylated in carbon tetrachloride (250 ml.) with methyl sulphate (40 ml.) and 30% NaOH (85 ml.). The mixture was stirred at 50° for 12 hr. and the methylated material separated by fractional crystallization of the insoluble matter by means of ethanol and acetone.

The product was further methylated with methyl iodide and $Ag_2O$. The final product (OMe, 30·5%) was separated into four fractions by the addition of light petroleum to a chloroform solution.

Two separate batches were methylated and thus eight fractions (OMe, 25·5–39·9%) were separated. Investigation showed that most of these contained a low percentage of sulphate residues and they were acid to congo red. In addition it was found impossible to increase their methoxyl content by further methylation, so that it was necessary to recombine them in order to remove the sulphate residues, part of which seemed to be present as ester sulphate groups. The sulphate was removed in the manner described below.

*Preparation of partly methylated degraded chondroitin.* The combined fractions in the form of a syrup (6 g.) were dissolved in a little water and 0·1 N-NaOH (150 ml.) added. The solution was heated on a boiling water-bath for 4½ hr., neutralized with dilute $H_2SO_4$, and concentrated in a vacuum to 50 ml. Dialysis, with a cellophan membrane, was shown to be ineffective as a method of removing the inorganic sulphate, since the carbohydrate passed through the membrane, and the following treatment was substituted. Excess of a neutral aqueous solution of lead acetate (analytical reagent) was added to the concentrated solution and the resulting precipitate of $PbSO_4$ removed (centrifuge). The Pb in solution was removed by means of $H_2S$, and the solution, filtered through a charcoal pad, was concentrated to a syrup which now gave no test for sulphate residues. It was further purified by repeatedly dissolving it in absolute ethanol, concentrating the solution and filtering off the inorganic material which separated.

*Preparation of fully methylated degraded chondroitin.* The above syrup, which was an acid, was dissolved in acetone containing a few ml. of dry methanol, and methylated and esterified by means of $Ag_2O$ and methyl iodide in the usual way, two treatments being given. The methylated product, now soluble in dry acetone, was isolated in the form of a clear syrup which contained some AgI.

*Hydrolysis of methylated degraded chondroitin.* A trial hydrolysis with 8% hydrogen chloride in methanol was carried out on a small quantity of the syrup (0·5 g.). The mixture was refluxed on a boiling water-bath. $\alpha_D$ +0·035° (initial value); +0·09° (1·5 hr.); +0·15° (4·15 hr.); +0·19° (5·9 hr.); +0·19° (7·9 hr.) (constant value). At the end of 8 hr. the solution was neutralized with $Ag_2CO_3$ and the filtered solution concentrated to a thin syrup. This was extracted with ether, giving an ether-soluble fraction and an ether-insoluble fraction. The process was then repeated on a larger scale.

*Investigation of the ether-soluble fraction.* The ether-soluble fraction was obtained as a clear mobile syrup. A sample portion (0·35 g.) was distilled in a high vacuum and gave the fractions shown in Table 2A.

*Isolation of 2:3:4-trimethyl-α-methylglucuronoside.* Fractions 1 and 2 (Table 2A) were separately dissolved in a little dry methanol, the solution saturated with $NH_3$ and kept at 0° for 2 days. They were then concentrated to syrups which, on drying, crystallized completely. The products were recrystallized from an ethanol-ether mixture, light petroleum being added until the solution just became cloudy. From both fractions fine needle-like crystals were obtained which melted at 182–183°, alone or in admixture with an authentic specimen of the amide of 2:3:4-trimethyl-α-methylglucuronoside. The fact that this product was obtained shows that the original polysaccharide must contain glucuronic acid as an end-group.

The remainder of the ether-soluble fraction was then distilled and the fractions shown in Table 2B were obtained.

*Investigation of ether-insoluble fraction.* This fraction (OMe, 31·8%) contained N. It was an extremely viscous, clear syrup, with an acid reaction.

This fraction was first treated with dry methanol containing acetic anhydride (5% of total weight) to acetylate the amino group. The mixture was kept for 24 hr. and then

Table 2. *Properties of the ether-soluble methylated fraction*

| Fraction | Weight (g.) | b.p. (bath temp.) | $\alpha_D^{18°}$ | OMe (%) | Description |
|---|---|---|---|---|---|
| | | | A. Distillation of a sample portion | | |
| 1 | 0·0564 | 120–125° (0·03 mm.) | 1·4490 | 48·8 | Pale brown mobile syrup |
| 2 | 0·1105 | 125–134° (0·04 mm.) | 1·4520 | 51·65 | Pale yellow mobile syrup |
| Residue | 0·1735 | Above 165° | — | 30·4 | Dark brown viscous syrup, soluble in ether |
| | | | B. Distillation of the remainder | | |
| 1 | 0·8840 | 115–125° (0·025 mm.) | 1·4522 | 50·45 | Mobile syrup |
| 2 | 0·1638 | 125–140° (0·02 mm.) | 1·4556 | 48·1 | Less mobile syrup |
| 3 | 0·1292 | 140–165° (0·02 mm.) | 1·4570 | 34·0 | Viscous syrup |
| Residue 4 | 0·4590 | Above 165° | — | — | Very viscous syrup |

a little water added to decompose the excess acetic anhydride. The solvent and acetic acid were removed by distillation under reduced pressure at 40°. The resulting syrupy product was more mobile and no longer acid. On distillation in a high vacuum a small amount of a very viscous distillate was obtained at 165–180° (0·015 mm.), which was nucleated with an authentic specimen of fully methylated *N*-acetyl-α-methyl-chondrosaminide. After the addition of a little ethyl acetate the material readily crystallized. Recrystallized from ethyl acetate the fine, needle-like crystals had a melting-point 179–180°, alone or in admixture with 3:4:6-trimethyl-*N*-acetyl-α-methylchondrosaminide.

The addition of a little ethyl acetate to the undistilled residue, and its inoculation with the same chondrosamine derivative as above, brought about further crystallization.

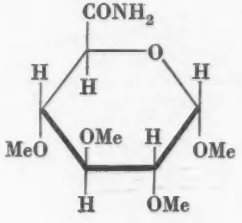
At this stage further investigation of the various fractions had to be suspended.

## RESULTS

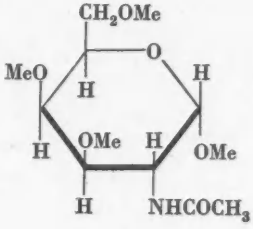
In all samples of chondroitin sulphate the amino group of the chondrosamine residues was acetylated. The product was difficult to acetylate and methylate fully and so was first degraded by hydrolysis with 0·1N-$H_2SO_4$ in order to give a product of lower molecular weight and to remove the sulphate residues. The hydrolysis liberated a small amount (4%) of amino sugar. The degraded product, for which the name 'degraded chondroitin' was retained, was acetylated with acetic anhydride and pyridine at room temperature. The acetylated degraded chondroitin was simultaneously deacetylated and methylated, initially with methyl sulphate and NaOH, and subsequently with methyl iodide and $Ag_2O$. The product was separated by precipitation into fractions which had methoxyl contents varying from 25 to 40%. In the earlier experiments it was found impossible to methylate further the fractions with lower methoxyl content, when methyl iodide and $Ag_2O$ were used. It was later discovered that the reason for this failure lay in the fact that the hydrolysis with dilute acid had not completely removed the sulphate residues. The carbohydrate derivative so produced, which contains both methoxyl groups and $H_2SO_4$ residues, appears to be novel and will undoubtedly be of importance when the allocation of the position of the sulphate residues is to be decided.

To complete the removal of sulphate groups the fractions were combined and again submitted to hydrolysis, this time with 0·1N-NaOH. The sulphate-free product was still acid to congo red, indicating that the carboxyl groups of part at least of the glucuronic acid units were not now engaged in linkage with the rest of the molecule. The syrup was then treated with $Ag_2O$ and methyl iodide to complete the methylation, and to esterify the free carboxyl groups present. During the purification of the fully methylated material some difficulty was encountered, due possibly to the formation of a complex between the AgI and the free amino groups in the polysaccharide, which had not been re-acetylated after the alkaline hydrolysis (cf. Irvine & Hynd, 1912; Stacey & Woolley, 1940). This complex was, however, decomposed when the methylated material was hydrolyzed with 8% hydrogen chloride in methanol.

Part (33%) of the material hydrolyzed on treatment with ammonia in methanol gave the crystalline amide of 2:3:4-trimethyl-α-methylglucuronoside (I) which was identical with an authentic specimen.




(I)




(II)

(where chondrosamine is assumed to be 2-aminogalactose)

The production of this fully methylated compound from the methylated polysaccharide establishes a number of facts. First, clear proof is furnished for the first time of the presence of glucuronic acid as a constituent of chondroitin, and secondly it proves that about one-half of the glucuronic acid residues must constitute terminal groups in the polysaccharide. Moreover, it is apparent that the linkage with the rest of the molecule must involve position 1 of these glucuronic acid residues, i.e. they are attached by glycosidic links.

The crystalline substance from the ether-insoluble fraction was identified as 3:4:6-trimethyl-*N*-acetyl-α-methylchondrosaminide (II), which had previously been prepared in this department from chondrosamine hydrochloride (cf. Levene, 1941), the yield indicating that about 33% of the product of hydrolysis of methylated degraded chondroitin consisted of *N*-acetyl chondrosamine which was

only partly methylated. The methoxyl content of this product indicated that it consisted mainly of a dimethyl-*N*-acetyl-methylchondrosaminide.

The viscous volatile fraction and the undistilled residue obtained from the distillation of the ether-soluble fraction had methoxyl contents of 30–34% and were N-free and acid in reaction. These have not yet been fully examined, but since they can be methylated further to give fully methylated glucuronic acid, the presence in them is suggested of a dimethyl glucuronic acid residue. (A dimethyl glucuronic acid requires OMe, 28%, and a dimethyl glucuronolactone, OMe, 30%.)

## DISCUSSION

At the stage which this investigation has reached it is possible to set forth some pertinent facts regarding the structure of chondroitin sulphate. Thus chondroitin sulphate occurs in cartilage in the form of a high molecular muco-protein from which the protein constituent can be removed by mild saponification. The protein-free carbohydrate is still a high polymer constituted of units of *N*-acetyl chondrosamine, glucuronic acid, and sulphuric acid. The last-named residues can be split off by hydrolysis with strong alkali or weak mineral acid. The acid hydrolysis causes some degradation with the liberation of a small percentage of a labile amino sugar, but the residual 'degraded chondroitin' is non-reducing to Fehling's solution. Its solutions, as well as those of its acetylated and methylated derivatives, have low viscosities and the compounds readily dialyze through cellophan membranes. Thus the molecular weight of degraded chondroitin is relatively low, the minimum possible being that of a trisaccharide.

From the isolation of a high percentage of terminal glucuronic acid groups it is clear that the molecule is of the branched chain type (Stacey & Woolley, 1942), and the carboxyl groups of some of the glucuronic acid units may be involved as polymeric links in chondroitin. A complete knowledge of the structure of chondroitin sulphate must await further study, particularly of the identities of the partially methylated chondrosamine and glucuronic acid portions. In this connexion a new aspect of the problem has been presented by the work of Percival & Soutar (1940) and Duff & Percival (1941), who have shown that the removal of sulphate groups from hexoses by means of $Ba(OH)_2$ gives rise to anhydro sugars. A search will need to be made for such compounds among the hydrolytic products of methylated chondroitin.

## SUMMARY

1. Chondroitin sulphate was isolated from the nasal septa of cattle and from bovine and human trachea.

2. It was degraded and methylated to give fully methylated, sulphate-free degraded chondroitin of relatively low molecular weight. From an acid hydrolysate, derivatives of glucuronic acid and chondrosamine were isolated and characterized as the amide of 2:3:4-trimethyl-α-methylglucuronoside and 3:4:6-trimethyl-*N*-acetyl-α-methylchondrosaminide respectively.

3. Three facts were established: first, glucuronic acid and chondrosamine are constituents of the molecule of chondroitin sulphate; secondly, part at least of the glucuronic acid residues is present as terminal groups; and thirdly, the linkage between these residues and the remainder of the molecule is of a glycosidic nature, involving $C_1$ of the glucuronic acid residues. Chondroitin sulphate possesses a structure of the branched chain type.

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# Quenching Effect of Electrolytes on the Fluorescence Intensity of Riboflavin and Thiochrome

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After the discovery of riboflavin, and its identification as vitamin $B_2$, assay methods based on the measurement of its fluorescence intensity were described (v. Euler & Adler, 1934; Cohen, 1934; Josephy, 1934). Similar methods of assay were developed for vitamin $B_1$ after Jansen (1936) had shown that thiochrome, the fluorescent oxidation product of thiamin, could be used as the basis for the fluorimetric estimation of this vitamin. These methods have been developed by numerous workers for use in the determination of the vitamin content of foodstuffs, tissues and body fluids. In all such assays the fluorescent intensity of riboflavin is measured in aqueous or butanol solution; thiamin is extracted, oxidized to thiochrome and measured fluorimetrically in *iso*butanol solution. The methods used for both vitamins (cf. papers quoted by Najjar, 1941, and the Report of the Vitamin $B_1$ Sub-Committee (Appendix A), Medical Research Council, 1943), do not prevent the appearance in the purified extract of a number of substances present in the original material, particularly since a considerable amount of water is soluble in butanol. Such substances might significantly influence the fluorescence intensity of riboflavin or thiochrome, and thus interfere with the accuracy of the assay. An investigation has therefore been made on the effect of a number of substances on the fluorescence intensity of the two fluorescent pigments. The quenching effect of oxygen, which was stressed by Weil-Malherbe & Weiss (1942), was not taken into account because all measurements were made in equilibrium with air and the standards used were equally affected.

## METHODS

### (*a*) *Preparation of riboflavin and thiochrome solutions*

*Riboflavin.* Solutions of riboflavin in water containing 100, 50, 20 and 2 mg./l. were diluted with equal amounts of 0·2 N solutions of various inorganic and organic compounds,* so that the riboflavin concentration of the solution to be measured was 50, 25, 10 and 1 mg./l., and the salt concentration 0·1 N. With $Na_2HPO_4$, NaCl, $FeCl_3$ and $FeSO_4$, solutions of other concentrations were also examined. To study the effect of pH on the fluorescence intensity, solutions of riboflavin in water containing 25 mg./l. were diluted with equal amounts of buffer solutions (Sörensen's borate and phosphate buffers; Michaelis's acetate and tartrate buffers) to obtain pH values between 11·2 and 2·4. The pH of each solution was measured in a glass-electrode pH meter. Only those salts which did not quench the fluorescence in the neutral region were used as buffers.

*Thiochrome.* Thiochrome was prepared from thiamin according to the procedure of Barger, Bergel & Todd (1935). The effect of the presence of quenching substances, and of changes in pH on the fluorescence of thiochrome dissolved in water or in non-fluorescent *iso*butanol was examined. The aqueous solutions were prepared by dilution of a solution of thiochrome containing 20 mg./l. with equal amounts of 0·2 N salt solutions. To find the effect on *iso*butanol solution two series of preparations were made. In one series *iso*butanol solutions of thiochrome of 5 and 2·5 mg./l. were saturated with aqueous 0·2 N salt solutions. The saturation point of *iso*butanol with water and salt solutions at room temperature was found to be approximately 10%. Therefore 9 parts of the *iso*butanol solutions were thoroughly shaken with 1 part of the aqueous salt solutions and kept at room temperature for about 18 hr. The fluorescence intensity of this solution was then compared with that of a dry *iso*butanol solution, and with that of one shaken with 10% of distilled water. In the other series conditions were chosen which correspond to those of some of the assay methods in use. Two ml. of an aqueous solution containing 10 mg./l. of thiochrome and a 0·1 N concentration of the salt were shaken with 5 ml. *iso*butanol for 3 min. The *iso*butanol layer was removed, dried with anhydrous $Na_2SO_4$, and its fluorescence intensity compared with extracts prepared in the same way from a thiochrome solution in distilled water.

The effect of changes in pH on the fluorescence of thiochrome in aqueous and *iso*butanol solutions was also investigated. The aqueous solutions were prepared, by methods similar to those used for riboflavin, from a thiochrome solution of 20 mg./l. The *iso*butanol solution was saturated with the corresponding aqueous buffer solution.

### (*b*) *Measurement of fluorescence*

The fluorescence intensity, excited by the 366 mμ line of a mercury lamp, was measured with a photoelectric fluorimeter designed by the authors (Ellinger & Holden, 1944), in which 2 mm. containers for the solutions were used. Most determinations were made by an electrical balancing method and some by a direct deflexion method (Ellinger & Holden, 1944). Quenching was determined on the basis of a comparison of the observed fluorescence intensity of the experimental solution with that expected from the known concentration of fluorescent material. The extent of quenching was expressed as a percentage of the fluorescence intensity of the pigments in solvents containing no quenching substances. The absorption of the 366 mμ line was measured for the riboflavin-salt mixtures with the same instrument.

* With uric acid, the solution was saturated.

## RESULTS

The results of the quenching effect of electrolytes and organic substances are given in Tables 1–3.

Table 1. *Quenching effect of various inorganic and organic compounds on the fluorescence intensity of aqueous riboflavin solutions of various concentrations*

($I_0$ = fluorescence of unquenched solution. $I$ = fluorescence of quenched solution.)

| Quenching substance | $100\,(I_0 - I)/I_0$ determined by Direct deflexion method for solutions of riboflavin containing (mg./l.) | | | $100\,(I_0 - I)/I_0$ determined by Electrical balancing method for solutions of riboflavin containing (mg./l.) | |
|---|---|---|---|---|---|
| | 50 | 10 | 1 | 10 | 25 |
| NaF | None | None | None | None | — |
| NaCl | — | 7·4 | 7·0 | 6·5 | 7·4 |
| NaBr | 65·9 | 65·8 | 65·5 | 68·2 | — |
| NaI | 85·8 | 85·4 | 87·5 | 85·5 | — |
| NaCN | 88·2 | 85·2 | — | 84·1 | — |
| NaCNS | 71·2 | 73·1 | 69·0 | 73·7 | — |
| $NaNO_2$ | 83·6 | 83·9 | — | 84·6 | — |
| $NaNO_3$ | 5·8 | 5·6 | 6·5 | 7·4 | — |
| $NaHCO_3$ | None | None | None | None | — |
| $Na_2SO_3$ | 45·2 | 44·7 | 43·7 | 42·0 | — |
| $Na_2SO_4$ | None | None | None | None | — |
| $Na_2HPO_4$ | 12·6 | 13·4 | 14·1 | 9·0 | — |
| Sodium acetate | None | None | None | None | — |
| Sodium citrate | None | None | None | None | — |
| Sodium tartrate | None | None | None | None | — |
| Sodium lactate | None | None | None | None | — |
| Urea | None | None | None | None | — |
| Uric acid | None | None | None | None | — |
| KCl | — | — | — | — | 6·9 |
| $NH_4Cl$ | — | — | — | — | 6·9 |
| $MgCl_2$ | — | — | — | — | 6·9 |
| $CaCl_2$ | — | — | — | — | 6·9 |
| $FeSO_4$ | — | — | — | — | 44·4 |
| $FeCl_3$ | — | — | — | — | 92·3 |

Table 2. *Quenching effect of various salt concentrations on the fluorescence intensity of riboflavin*

(Quenching is calculated from $(I_0 - I)/I_0 \times 100$: $I_0$ = fluorescence of unquenched solution; $I$ = fluorescence of quenched solution.)

| Quenching substance | Quenching with solutions containing quenching substances in final concentration (normality) | | | | | |
|---|---|---|---|---|---|---|
| | 0·20 | 0·10 | 0·05 | 0·02 | 0·01 | 0·005 |
| NaCl | 23·1 | 6·9 | — | 2·4 | 1·0 | — |
| $Na_2HPO_4$ | — | 13·1 | — | 5·9 | 3·0 | — |
| $FeCl_3$ | — | 92·3 | 83·3 | 57·2 | — | 25·9 |
| $FeSO_4$ | — | 44·4 | 31·6 | — | — | 5·9 |

Table 3 shows that distilled water has a quenching effect on thiochrome in dry *iso*butanol. The values of quenching obtained for the salts allow for the quenching effect of the water dissolved in the *iso*butanol. In column 5 of Table 3 the quenching values are based on an average yield of 82% for the extraction of thiochrome from distilled water by *iso*butanol under the conditions of these experiments. The values for the percentage of quenching in this column are related to this reduced value. The shaking of the *iso*butanol with aqueous thiochrome solutions containing $MgCl_2$ or $CaCl_2$ resulted in the formation of an emulsion which was broken slowly. The values given in the last column of Table 3 were obtained after these solutions had stood for 18 hr. If measurements were carried out without complete separation of the layers, quenching up to 30% was observed.

The measurement of the absorption of light of wave-length 366mμ by the various salt solutions showed that absorption was increased by about 60% in the presence of $NaNO_2$ and by a very small extent in that of NaI. All the other substances did not affect the absorption.

The effect of variation in pH upon the fluorescence intensity of thiochrome and riboflavin is given in Fig. 1.

## DISCUSSION

From Table 1 it is evident that a considerable quenching effect on riboflavin fluorescence is observed only with a number of anions and with iron salts (ferrous and ferric), and that the quenching effect does not depend on the concentration of riboflavin. The observations parallel those of Eisenbrand (1934) on the quenching effect of various salts on the fluorescence intensity of quinine sulphate; he found that cations have little or no effect, while, of the anions, the effect of the halogens is important and increases with increasing atomic number. Of the anions the halides, cyanide, thiocyanide, sulphite and nitrite have a considerable effect on the fluorescence intensity of riboflavin. That of nitrite is mainly due to increased absorption of the primary beam. The effect of the chlorides of Na, K, $NH_4$, Mg and Ca is due to the anion. The considerable effect of ferrous and ferric ions may be explained as due to a reversible oxidation-reduction process, as observed by Weiss (1938) for the quenching of the fluorescence of certain dyes by iron ions. The quenching effect of phosphate and bicarbonate, and in some measure of cyanide, is due to the alkalinity of the solutions containing these anions. Neutral phosphates do not quench. Table 2 shows that the quenching effect is a function of the concentration of the quenching substance.

The quenching effect of electrolytes on thiochrome fluorescence is more complex. In aqueous solution considerable quenching occurs only with halides, nitrite, ferricyanide, lactate, $NH_4$ and iron salts. In the halide series the fluorides and iodides have a much higher quenching effect than chlorides and bromides. The effect of the nitrite ion is due to its

Table 3. *Quenching effect of inorganic and organic compounds on the fluorescence intensity of thiochrome solutions*

(Quenching is calculated from $(I_0 - I)/I_0 \times 100$, where $I_0$ = fluorescence of unquenched solution, and $I$ = fluorescence of quenched solution.)

| Quenching substance | Quenching observed in: Aqueous solution containing 0·1 N quenching substance and 10 mg./l. of thiochrome | *Iso*butanol solution saturated with 0·2 N solution of quenching substance. Concentration of thiochrome (mg./l.) 5 | 2·5 | *Iso*butanol extract of an aqueous solution of thiochrome containing 0·1 N quenching substance and 10 mg./l. of thiochrome |
|---|---|---|---|---|
| $H_2O$ | — | 4·7 | 5·0 | — |
| NaF | 28·5 | 22·7 | 21·7 | 11·5 |
| NaCl | 5·0 | 16·0 | 17·5 | 10·1 |
| NaBr | 4·0 | 22·2 | 23·0 | 7·4 |
| NaI | 20·5 | 39·0 | 39·8 | 24·2 |
| NaCN | None | 9·5 | 8·8 | None |
| NaCNS | None | — | 7·1 | 6·0 |
| $NaNO_2$ | 57·0 | 25·7 | 26·9 | 8·8 |
| $NaNO_3$ | None | 12·3 | 10·1 | 7·5 |
| $Na_2CO_3$ | None | — | — | None |
| $Na_2SO_3$ | None | 5·2 | 5·1 | None |
| $Na_2SO_4$ | None | 12·3 | 11·3 | None |
| $Na_2HPO_4$ | None | 9·1 | 8·4 | None |
| Sodium acetate | None | 8·7 | 7·7 | None |
| Sodium citrate | None | — | 6·8 | None |
| Sodium tartrate | None | 6·6 | 7·4 | None |
| Sodium lactate | 43·9 | — | 7·1 | 1·2 |
| Urea | None | 9·1 | 8·7 | 5·8 |
| Uric acid | None | 8·5 | 8·0 | 8·3 |
| $K_3Fe(CN)_6$ | 100 | — | 19·2 | 25·4 |
| $K_3Fe(CN)_6$ (0·02 N) | 100 | — | — | 17·9 |
| KCl | 8·7 | 18·7 | 18·3 | 10·1 |
| $NH_4Cl$ | 23·6 | 18·4 | 18·0 | 6·5 |
| $MgCl_2$ | 6·4 | 20·6 | 21·4 | 0·3 |
| $CaCl_2$ | 6·4 | 24·0 | 22·3 | 1·6 |
| $FeCl_3$ | 88·4 (0·04 N) | 81·8 (0·04 N) | 80·0 | 33·2 |
| $FeCl_3$ | 100·0 | 88·9 | — | 96·9 |

NOTE. Bracketed values refer to concentrations of salt used in these instances.

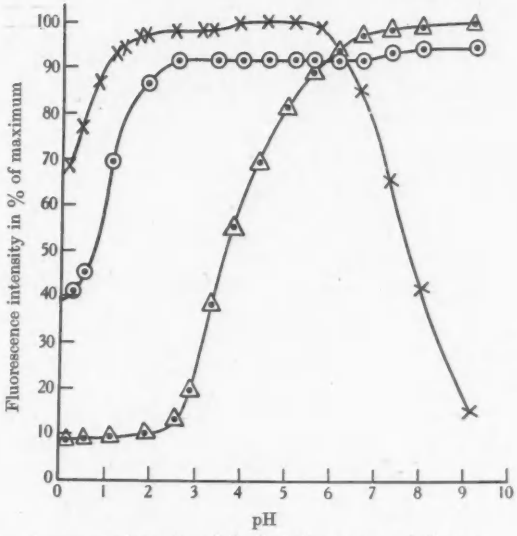


Fig. 1. Effect of pH on fluorescence intensity of riboflavin in aqueous solution ×——×; and of thiochrome in aqueous solution △——△ and in *iso*butanol ⊙——⊙.

absorption of the exciting light. The high quenching effect of lactate is remarkable, and requires further investigation for its explanation.

The quenching effect in *iso*butanol solutions is influenced by the absolute and relative solubilities of the quenching compounds in water and *iso*butanol. In the saturated *iso*butanol solution it is higher for most substances than in aqueous solution. For a number of substances which do not quench in aqueous solution, slight quenching occurs in *iso*butanol saturated with water, but for a number of substances, particularly chloride, bromide and iodide, the quenching in *iso*butanol is considerably greater than in aqueous solution. The effect of potassium chloride is probably due to the chloride ion; the slightly greater one of Mg and Ca may be partly due to the cation. The quenching effect seen in the *iso*butanol extracts of the aqueous solutions is governed by the partition coefficient of the quenching substance between water and *iso*butanol.

Apart from the quenching effect of the halide and the slight effect of some others, the considerable quenching by the ferric cation and the ferricyanide anion is remarkable. Potassium ferricyanide in a concentration of 0·02N has a quenching effect of 18%. Since this substance is used in the preparation of thiochrome for the assay of vitamin $B_1$ the concentration of this salt in the final extract may be of great influence.

The curve of the dependence of the fluorescence intensity of riboflavin on the pH shows a maximum between pH 5·9 and 7·7; between 3·8 and 5·9 the intensity is about 97% of the maximum. At pH values below 3·8 and above 7·7 there is a rapid fall in the fluorescence intensity. The curve corresponds to that published by Kuhn & Moruzzi (1934).

With thiochrome in aqueous buffer solutions the maximum fluorescence intensity is reached at the alkaline end of the range examined (pH 11). The intensity decreases slowly at pH 8·8, then more rapidly at pH 7·5 and falls steeply at pH 5. This is in accordance with the findings of Kuhn & Vetter (1935). Below this pH the blue fluorescence has completely disappeared and there is only a small amount of green fluorescence. In *iso*butanol solutions saturated with buffer solutions the effect is less marked. There is very little alteration between pH 11 and 4·5, but below 4·5 the fluorescence intensity falls steeply.

## SUMMARY

The influence of changes in pH of the solution, and of the presence of a number of inorganic and a number of organic compounds on the fluorescence intensity of riboflavin and thiochrome, has been investigated.

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# A Method for the Estimation of Nicotinamide Methochloride in Urine

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Najjar & Wood (1940), Najjar & Holt (1941 *a*, *b*) and Najjar, Stein, Holt & Kabler (1941) described a fluorescent pigment '$F_2$' in the urine of normal dogs and of man, the elimination of which increased after ingestion of nicotinamide and related compounds, but which was absent from the urine of pellagrins. It was suggested by Coulson, Ellinger & Platt (1942) that information about the metabolism of these compounds could be expected from an investigation of the urinary elimination of $F_2$, and indeed a preliminary method of assay was developed for this specific purpose before the chemical

nature of the compound was known (Coulson, Ellinger, Glock & Platt, 1942). A large number of estimations was made with this method, which provided relative but not absolute values. The investigation of the nature of $F_2$ led Huff & Perlzweig (1943*a*) to state that the fluorescent $F_2$ had the same absorption spectrum as, and was identical with, nicotinamide methochloride. Ellinger & Coulson (1943) and Coulson & Ellinger (1943), however, showed that synthetic nicotinamide methochloride (1-methylpyridinium chloride-3-carboxylic acid amide) is not fluorescent and only becomes fluorescent on treatment with alkali and transfer into *iso*butanol. They showed further that the substance which is found in urine in increased amounts after ingestion of nicotinamide is also non-fluorescent until treated with alkali and *iso*butanol. The natural untreated substance was then isolated and identified as nicotinamide methochloride. It was suggested that $F_2$ formed *in vitro* was most probably a mixture of $F_{2b}$, 6-hydroxy-1:6-dihydro-1-methylpyridine-3-carboxylic acid amide, with an absorption maximum at 358 m$\mu$, and another carbinol $F_{2a}$ with an *o*-quinoid structure and three absorption maxima at 271, 306 and 347 m$\mu$. The elucidation of the nature of nicotinamide methochloride and of the mechanism of the fluorescence development provided a means of improving the assay so that absolute values could be obtained. While this work was in progress two methods of assay were published by Huff & Perlzweig (1943 *b*, *c*). With regard to the nomenclature it should be mentioned that the non-fluorescent precursor should be called nicotinamide methochloride, since the crystalline form on which the measurement is based is a chloride. The fluorescent derivatives should be called '$F_2$' until their structure is fully proved by further experimental evidence.

## METHOD OF ASSAY

The principle of the method is based on the separation of nicotinamide methochloride from urine by adsorption and elution, transformation of the substance into fluorescent derivatives ($F_2$) by treatment with alkali and extraction with *iso*butanol, and estimation of the concentration of $F_2$ in *iso*butanol by visual fluorimetry.

The assay is carried out as follows. The lower ends of thistle funnels of 4–5 mm. internal diameter are plugged with glass wool, and 1 g. Decalso (60 mesh) (Permutit Co., Ltd.) is washed into each tube. Five ml. or less of the urine diluted to 10 ml. are filtered through the column, which is then washed with 50 ml. distilled water and eluted with 14 ml. 25% (w/v) KCl solution. The eluate is collected in a dry tube and can be stored in the cold without deterioration. The eluate is divided into two equal portions which are transferred into glass-stoppered tubes of about 12 ml. capacity. Non-fluorescent *iso*butanol (2 ml.) is added to each and 1 ml. 15% (w/v) NaOH to one to develop fluorescence. The tubes are shaken at once vigorously for 5 min., and after separation of the layers the *iso*butanol is pipetted off into identical non-fluorescent test-tubes, each containing about 100 mg. anhydrous $Na_2SO_4$.

The *iso*butanol extract of the neutral half of the KCl-eluate is used as a reagent blank. The fluorescence intensity of the extract is compared with that of standards. The extracts are stable at room temperature in the dark.

Standards are prepared by passing a series of 10 ml. portions containing 1–12 $\mu$g./ml. of crystalline nicotinamide methochloride, prepared as described by Karrer, Schwarzenbach, Benz & Solmssen (1936), in distilled water, through 1 g. Decalso columns. The columns are washed and eluted with 14 ml. 25% KCl solution in the manner described above. Seven ml. of each of the eluates are made alkaline and extracted with 2 ml. *iso*butanol. The separated extracts, dried with $Na_2SO_4$, are used as the standards. The tubes should be identical with those used for the extracts in the assay and the standards should be kept in the dark.

The *iso*butanol extracts are read in the filtered 366 m$\mu$ line beam of a high-pressure mercury lamp by naked-eye comparison of the unknown with the standards, in a dark room. With such standards no instrument for measuring fluorescence intensity is necessary apart from the high-pressure mercury lamp. To increase the accuracy of the assay the results of three estimations with different amounts of urine should be averaged.

For large-scale routine work wooden racks supporting twelve thistle funnels were used. Sixteen tubes were shaken simultaneously in a box constructed for the purpose.

### *Examination of the various steps involved in the assay*

*Stability of nicotinamide methochloride in urine.* Nicotinamide methochloride in urine was found to be stable for at least a week at room temperature in the dark in spite of the decomposition of the urine. It was therefore possible to collect specimens in distant districts and assay them, without loss, in the laboratory at a convenient time. Substances generally used for preserving urines interfere with the various steps of the procedure and should not be used. Boiling of urine for 15 min. does not affect the assay.

*Adsorption.* Of a number of adsorbents examined Decalso was found to be the most satisfactory from the standpoint of adsorption and elution. The speed of filtration, an important factor in large-scale experiments, is satisfactory with samples of about 60 mesh. The disadvantages of this adsorbent are that it cannot be sufficiently reactivated for further use, and that different batches may have a different physical and chemical constitution and therefore different adsorptive properties. Decalso of the same batch should be used for assay and preparation of standards. Up to 1 mg. of nicotinamide methochloride in 10 ml. is completely adsorbed from aqueous solutions by 1 g. Decalso. The adsorption is affected by electrolytes in concentrations above 0·4 M but not by urea or changes of pH within the range 3–8. Five ml. of urine should be used for the assay if the concentration of nicotinamide methochloride is less than 12 $\mu$g./ml. and the hourly urine volume is greater than 100 ml. With higher concentrations and lower urine volumes, correspondingly smaller amounts of urine must be taken and diluted to 10 ml. to reduce the danger of incomplete adsorption.

*Washing.* Washing with distilled water is essential to remove all materials which might interfere with the assay, particularly fluorescent pigments contained in the original

urine. Fifty ml. of water are always sufficient for this purpose.

*Elution.* Elution by the chlorides of Na, K, Ca, Sr and Ba was investigated. KCl in a concentration of 25% was found to be the most efficient. The chlorides of the bivalent cations gave almost unbreakable emulsions when shaken with *iso*butanol. The elution of the nicotinamide methochloride is an equilibrium process and is not complete when 14 ml. 25% KCl are used for 1 g. Decalso under these conditions. Two-thirds of the total adsorbed material is always eluted, as shown in Table 1.

Table 1. *Relative fluorescence intensities of two series of* iso*butanol extracts of successive* KCl *eluates from the same Decalso columns*

| No. of elution | Relative intensities of fluorescence | |
|---|---|---|
| 1 | 100 | 100 |
| 2 | 29 | 33 |
| 3 | 9·7 | 10 |
| 4 | 3·1 | 2·8 |

*Conversion and extraction.* The conversion of nicotinamide methochloride into fluorescent derivatives, and the extraction of these derivatives, are also equilibrium processes. In aqueous alkaline solution the equilibrium favours the quaternary ammonium base and very little of the fluorescent derivatives is present. In *iso*butanol the fluorescent substances which are readily soluble in water and in *iso*butanol are predominant (Ellinger & Coulson, 1943). In the assay the mixture of the two fluorescent derivatives can be treated as a single pigment for practical purposes. An aqueous alkaline solution of nicotinamide methochloride is unstable. In the first 10 min. after the addition of alkali the deterioration is negligible, but standing for 12 hr. completely destroys the substance. The extraction should be carried out as quickly as possible after the NaOH has been added to the eluate, to avoid the risk of loss by destruction. For optimal extraction vigorous shaking by hand or mechanical shaker for not less than 5 min. is essential. Longer shaking or high speed mechanical shaking does not increase the yield.

In order to compare the intensity of the fluorescence derived directly from nicotinamide methochloride with that of the standards which had been adsorbed on Decalso, an *iso*butanol stock solution of the fluorescent derivatives was prepared in the following way. Ten mg. nicotinamide methochloride dissolved in 2 ml. of water were diluted to 100 ml. with non-fluorescent *iso*butanol. A pellet of NaOH was added and the solution shaken for about 5 min. until no further increase in fluorescence was observed. After standing for ½ hr. in the dark the remainder of the NaOH pellet was removed. Solutions containing 0·025–0·5 μg./ml. at narrow intervals were prepared by dilution of this stock solution. The diluted solutions were then fluorimetrically compared with the standards used in the assay. The results are shown in Fig. 1.

If we assume that nicotinamide methochloride in *iso*butanol is converted quantitatively into the fluorescent derivatives, an *iso*butanol solution of 0·5 μg./ml. corresponds in fluorescence intensity to that of the 2 ml. *iso*butanol extract derived from an aqueous or urine solution which contained 12·12 μg. and was treated in the manner of the assay of nicotinamide methochloride. The 2 ml. *iso*butanol extract derived from the above urine would contain 1·0 μg. of the $F_2$. This represents an over-all yield of 16·5%, since only one-half of the KCl eluate is used for the assay.

Under the conditions of the assay (7 ml. 25% KCl, 1 ml. 15% NaOH and 2 ml. *iso*butanol), one single extraction

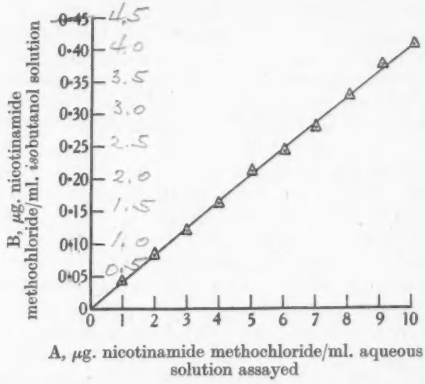


Fig. 1. Relation of fluorescence intensity obtained from 10 ml. solutions of nicotinamide methochloride that have undergone the assay (A) to that of $F_2$ solutions developed directly by alkaline *iso*butanol treatment of nicotinamide methochloride (B).

transfers 45% of the pigments extractable into the *iso*butanol phase. If the eluate is extracted with four successive 2 ml. portions the aqueous solution is practically exhausted. The yields of four successive extracts are shown in Table 2.

Table 2. *Relative yield of fluorescent derivatives of nicotinamide methochloride from four successive* 2 *ml.* iso*butanol extractions of* 7 *ml.* 25% KCl *eluates*

(Eluates obtained from 0·3–3·0 ml. of same 23 hr. urine sample after an intake of 500 mg. nicotinamide.)

| Vol. urine used (ml.) | Fluorescence intensity (arbitrary units) of extract | | | | Fluorescence intensity of first extract in % of the total* |
|---|---|---|---|---|---|
| | First | Second | Third | Fourth | |
| 0·3 | 2·1 | 2·1 | 0·2 | 0·06 | 47·1 |
| 0·5 | 3·5 | 2·5 | 0·9 | 0·0 | 50·7 |
| 0·8 | 5·8 | 5·8 | 1·5 | 0·06 | 44·0 |
| 1·0 | 6·8 | 6·3 | 1·5 | 0·06 | 46·3 |
| 2·0 | 12·0 | 12·0 | 2·8 | 0·1 | 44·6 |
| 3·0 | 15·5 | 15·0 | 4·0 | 0·8 | 43·3 |

* Values of column 6 are obtained by dividing the values of column 2 by the sums of columns 2–5 and multiplying them by 100.

The final yield after the processes of elution, conversion and extraction is, as shown, about 16·5%. Since the loss in elution is 33%, and that in extraction 55%, then by calculation the loss in conversion is about 45%.

To render the *iso*butanol non-fluorescent it is sometimes necessary to remove any fluorescent pigments present by shaking with charcoal; norite or activated charcoal (British Drug Houses, Ltd.) were found to be suitable for this

purpose. Contact of *iso*butanol with corks that have not been exhaustively extracted with *iso*butanol must be avoided.

*Measurement of fluorescence intensity.* The molar fluorescence intensity of $F_2$ in *iso*butanol is relatively low, being only about one-third that of quinine sulphate in 0·1 N-$H_2SO_4$ and one-ninth that of thiochrome in *iso*butanol. The fluorescence efficiency in the 366 mμ line (intensity when 50% of the incident light is absorbed) is low, about one-tenth that of quinine sulphate in 0·1 N-$Na_2SO_4$ and one-sixth that of thiochrome in *iso*butanol. Therefore only a comparatively narrow range can be measured with sufficient accuracy. Between 0·025 and 0·5 μg./ml. of the fluorescent derivatives can be estimated with the naked eye and 0·1–10 μg./ml. with the photoelectric fluorimeter of Ellinger & Holden (1944), with a 2 mm. cuvette. The fluorescence intensity in *iso*butanol is not affected by pH in the range between 9·24 and 3. Of a number of inorganic substances which are used in the assay, or which might be present in the urine, a slight quenching effect was found only with $Mg^{++}$, $Ca^{++}$, $SO_4^{--}$ and $SO_3^{--}$. This quenching effect, however, is negligible with the concentrations of these substances likely to be encountered.

The measurement of the fluorescence intensity is carried out preferably by a visual method, first, on account of the higher speed and secondly, because lower concentrations are measurable. By this method it is possible to detect a difference of 0·15 μg./ml. of nicotinamide methochloride in the range between 0·6 and 6 μg./ml. of original aqueous solution and a difference of 0·3 up to 12 μg./ml. of solution. If the concentration in the final extract is higher than that derived from 12 μg./ml., accurate reading is impossible. Fairly accurate estimation can be obtained by dilution of the *iso*butanol extract until it is within the range of the standards; it is, however, preferable to repeat the assay with a smaller amount of urine to safeguard against the possibility of incomplete adsorption.

Fresh standards should be prepared each week in sealed tubes and kept cool in the dark. The fluorescent pigments are unstable in the long wave ultra-violet region of mercury or daylight. The repeated short exposures in the usual readings of one week, do not, however, materially affect the fluorescence intensity.

## RESULTS

To test the accuracy of the method, various concentrations of nicotinamide methochloride in urine of known low $F_2$ content were estimated. The results are shown in Fig. 2.

It is seen that accurate estimations are possible for any concentration greater than 0·6 μg./ml. Between 0·6 and 1·0 μg./ml. the accuracy is about 10% and from 1·0 μg./ml. upwards the accuracy is about 5%. Discrimination of small differences in fluorescence intensity is best in concentrations between 2 and 5 μg./ml. From Fig. 2 it is evident that the nicotinamide methochloride estimations of aqueous solutions are more accurate (0·4% mean error) than those of urine solutions (5% mean error). The values from urine are consistently lower than calculated. Experiments are in progress to reduce this error and to determine whether it is due to incomplete adsorption caused by the presence of electrolytes or to some quenching substances accompanying nicotinamide methochloride through the assay procedure.

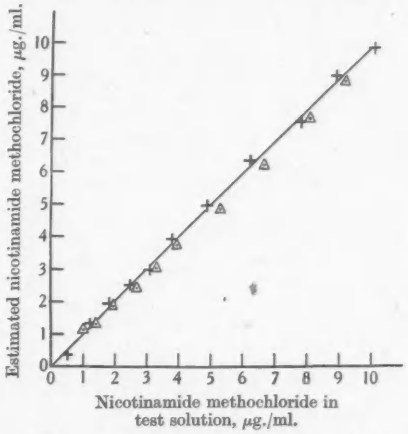


Fig. 2. Values obtained for a series of known nicotinamide methochloride concentrations in water (+) and in urine (△). The straight line represents the calculated values.

## DISCUSSION

The method has been evolved to make it possible to assay a large number of urine samples at the same time. Sixty single examinations can be carried out in one day by two workers. The two methods published by Huff & Perlzweig (1943 *b*, *c*) do not seem to have been developed for large-scale surveys.

With the method described, the daily output of nicotinamide methochloride was determined for normal persons and for experimental subjects who had taken nicotinamide and related compounds. Details of these experiments will be reported later. The daily output varies greatly in different individuals and in the same person at different times. From 2 to 8 mg./day are eliminated by normal persons. Somewhat lower results (0·1–3·2 mg. in 14 hr.) are reported by Sarett (1943). Variations are also observed in the nicotinamide methochloride urinary output of different subjects after the ingestion of nicotinamide. There is an average recovery of about 10–15% after the ingestion of 100 mg. nicotinamide. Continued administration leads to a gradual increase in the yield of nicotinamide methochloride in the urine. By repeated estimation of the daily output after successive administrations of nicotinamide a 'saturation test' can be obtained.

## SUMMARY

A method is described for the routine estimation of nicotinamide methochloride in urine. Diluted urine is passed through Decalso to adsorb the nicotinamide

methochloride. The Decalso is eluted with KCl solution, which is then made alkaline and extracted with *iso*butanol. The fluorescence produced is compared with that of standards similarly treated.

This investigation forms part of an investigation of nicotinamide deficiency carried out on behalf of the Air Ministry. We wish to thank Air Marshal Sir H. E. Whittingham, K.B.E., K.H.P., Director-General of the Medical Services of the Royal Air Force, for facilities provided. Our thanks are also due to L.A.C.W. A. E. Wrigglesworth for technical assistance. One of the authors (R. A. C.) is a member of the Civilian Technical Corps (Air Ministry), attached to the Lister Institute for an investigation of nicotinamide deficiency. The authors wish to thank Roche Products, Ltd., for a generous supply of nicotinamide.

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# Enzyme Formation and Polysaccharide Synthesis by Bacteria

## 2. POLYSACCHARIDE FORMATION BY *RHIZOBIUM RADICICOLUM* STRAINS

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An investigation into polysaccharide production by various strains of *Rhizobium* growing on the basal culture medium of Cooper, Daker & Stacey (1938) is described. The strains used were provided by Dr Hugh Nicol, and it was found that his two clover strains termed 'Clover Bartel A' and 'Clover 2027' were remarkably prolific in polysaccharide production. In general the purified polysaccharides gave laevo-rotatory, viscous solutions in water, although some differences in properties and yields of the polysaccharide were apparent from strain to strain. About 200 g. of the polysaccharide were obtained from the Bartel A strain.

In order to prevent contamination of the polysaccharide with the agar medium some modifications to the normal cultural conditions were made. For example, the usual agar culture medium, distributed in large Petri dishes, was covered with a layer of sterile cellophan according to the method of Birch-Hirschfeld (1933–4) and the surface inoculated with the bacterial suspension. The polysaccharide, uncontaminated by agar, was produced on the cellophan surface as a white mucilaginous growth which could easily be washed off and purified. In another method, suggested by Prof. W. N. Haworth, F.R.S., thick porous plates were embedded in agar medium so that nutrient material could freely diffuse through and be utilized by the organisms inoculated on to the surface of the plates. Polysaccharide production was good. In a third method the agar in the basal medium was replaced by acid-free kieselguhr, to provide a pasty solid medium which was distributed in Roux bottles and conical flasks. The organism grew well on the medium, covering the surface with a white mucilaginous material from which the polysaccharide could be obtained by extraction with water. The product made in this way generally had a much higher ash content than that made by other methods.

By an experimental procedure involving an examination of the hydrolysis products of its methylated derivative, which will be described elsewhere, part of the structure of the Clover Bartel A polysaccharide has been determined. The hydrolysis products were 2:3:6-trimethylglucopyranose (1 part), 2:3-dimethylpyranose (1 part) and 2:3-dimethylglucuronic acid (1 part). The type of structure assigned to the repeating unit is that of a polymerized aldobionic acid in which the carboxyl groups of the glucuronic acid residues are free. It has been shown previously (Cooper *et al.* 1938) that *Rhizobia* polysaccharides belong to the same class of compound as the specific polysaccharides from types II and III *Pneumococcus* (see Heidelberger & Goebel, 1927; Hotchkiss & Goebel, 1937). The structure of the latter has been worked out in some

detail by Reeves & Goebel (1941), and it may be formulated as follows:

$$\frac{4}{}\text{glucose}\frac{1:3}{}\text{glucuronic acid}\frac{1:4}{}\text{glucose}\frac{1:2}{}\text{glucuronic acid}\frac{1}{},$$

in which the numerals indicate the points of attachment.

In view of the precipitin reaction given in high dilution by the *Rhizobium* polysaccharide with anti-*Pneumococcus* horse serum type III and of the known close relationship between structure and serological specificity (Heidelberger, Kabat & Shrivastava, 1937; Heidelberger & Hobby, 1942), it may well be that the aldobionic acid units in the *Rhizobium* polysaccharide are of the cellobiuronic acid type. The function of the polysaccharide may be that of a defence mechanism against soil Protozoa and fungi.

## EXPERIMENTAL

### *Large-scale production*

For large-scale production the following medium described by Cooper *et al.* (1938) was used: $K_2HPO_4$ 1·0 g., asparagine 1·0 g., caffeine 0·2 g., NaCl 0·2 g., $MgSO_4.7H_2O$ 0·2 g., $FeCl_3$ 0·01 g., $MnCl_2$ 0·01 g., $CaCO_3$ 6·0 g., agar 1·8 g., sucrose 80·0 g. These were dissolved in 1 l. of lucerne root extract (made by boiling 10 g. of powdered lucerne root in 1 l. of tap water for 1 hr.). After one steaming the hot medium was transferred in 200 and 100 ml. amounts into Roux bottles, which were then subjected to two further steamings on successive days. Test-tubes containing 10 ml. amounts were also prepared. These were inoculated with the organism and, after an incubation period of 3 days at room temperature, 15 ml. of sterile water were added. 2 ml. of this bacterial suspension were transferred under sterile conditions to the Roux bottles. The bottles were incubated at room temperature for 10 days and the heavy yield of the gum was removed by cautiously skimming it from the solid agar surface after addition of a few ml. of distilled water. The resulting viscous solution was filtered through cotton-wool and run into twice its volume of ethanol, which was stirred continuously. The polysaccharide collected round the rod as a fibrous mass. The solvent was pressed out and the product dried under absolute ethanol and ether and in a vacuum. Thus obtained the polysaccharide had the appearance of asbestos fibre. For purification purposes the 'kieselguhr filtration method' as described by Cooper *et al.* (1938) was applied. This process removed the bulk of the bacterial debris.

### *Investigation on various strains of* Radicicola *species*

The organisms received from Dr H. Nicol were grown on the medium by the method of Cooper *et al.* (1938). Some of the strains—for instance those of the soya bean—failed completely to grow on this medium while other strains gave the yields shown in Table 1. Thus Clover Bartel A and Clover 2027 gave the optimum yields and of these Clover Bartel A was chosen for large-scale production since it had less ash than Clover 2027. Clover Bartel A gave a precipitin reaction in dilutions up to 1 in 100,000 with types III and VI anti-*Pneumococcus* horse sera (Dr M. Heidelberger, private communication).

Table 1. *Polysaccharide production by* Rhizobium

| Strain of *Rhizobium radicicolum* | Average yield of crude polysaccharide (g./l. of medium) | Ash (%) | $[\alpha]_D$ in water (corrected for ash) |
|---|---|---|---|
| West Australian lupin | 4·0 | 23·4 | −22° |
| Lucerne A and H | 2·5 | 57·2 | −14° |
| Pea 313 | 5·6 (mainly protein) | — | — |
| Pea B33 | 6·2 | 73·0 | −16° |
| Clover Bartel A | 7·5 | 29·5 | −14° |
| Clover Coryne | 5·6 (mainly protein) | — | — |
| Clover 202 | 2·1 | 28·0 | −17° |
| Clover 205 | 0·3 (mainly protein) | — | — |
| Clover 2027 | 7·5 | 38·9 | −14° |

### *Investigations on polysaccharides obtained from various Clover strains*

*Product from strain Clover Bartel A.* The crude polysaccharide (68·7% ash) gave very opalescent solutions in water, and polarimetric readings could not be taken. The solution was therefore acidified with a few drops of conc. HCl, and the polysaccharide reprecipitated by running the solution with continuous stirring into ethanol (2 vol.). This process was repeated, and finally the polysaccharide was precipitated from a neutral aqueous solution. This partially purified material contained 29·5% ash.

On hydrolysis with 0·5 N-$H_2SO_4$ the crude material gave the following readings: $[\alpha]_D^{20°}$, −10·4° (initial); −9·5° (15 min.); −6·6° (45 min.); −2·8° (75 min.); +1·9° (135 min.); +5·6° (240 min.); +9·5° (360 min.); +10·4° (420 min.); +12·3° (540 min.); +12·3° (600 min.). The hydrolysate gave, with naphthoresorcinol, a positive test for uronic acid residues.

*Product from strain Clover* 202. The polysaccharide, after reprecipitation twice from dilute HCl and once from a neutral solution by addition of ethanol, contained 28% ash. On hydrolysis with 0·5 N-$H_2SO_4$ the following readings were obtained: $[\alpha]_D^{20°}$, −11·1° (30 min.); −3·1° (60 min.); +1·0° (90 min.); +13·1° (180 min.); +17·2° (240 min.); +18·2° (300 min.); +21·2° (540 min.). The hydrolysate gave a positive naphthoresorcinol test for uronic acid residues.

*Product from strain West Australian Lupin.* This polysaccharide was hydrolyzed by 0·5 N-$H_2SO_4$, during which the rotation changed from $[\alpha]_D^{20°}$, +21·9° to $[\alpha]_D^{20°}$, +26·3° (equilibrium value after 450 min.).

*Product from strain Clover Coryne.* The initial precipitate disappeared completely when reprecipitation from an acid solution was attempted, showing that the material obtained consisted mainly of bacterial protein.

*Product from strain Clover* 2027. After repeated purification as described above, the polysaccharide contained 38·9% ash. On hydrolysis with 0·5 N-$H_2SO_4$ the following readings were obtained: $[\alpha]_D^{20°}$, +19·8° (3 hr.); +24·8° (4½ hr.). The naphthoresorcinol test for uronic acid residues was positive.

*Experiments on the prevention of agar contamination of the polysaccharides*

*Cellophan paper method.* Agar medium (75 ml.) was poured into each of six large Petri dishes and, after they had cooled, sterile cellophan paper was transferred on to the agar surface by means of sterile forceps, so that the whole surface was covered with cellophan. The dishes were then inoculated with a heavy dose of the usual bacterial suspension of *Rhizobium radicicolum* Bartel A and kept at room temperature for 8 days. The growth which had formed on the cellophan was then scraped off with a glass rod, the cellophan rinsed with distilled water and, after filtration through cotton-wool, the polysaccharide was precipitated in the usual way. Yield, 3·6 g./l. of medium; ash, 15·1%; N, 3·7%.

*Porous plate method.* A large crystallization dish was filled with 75 ml. of agar medium and a sterile porous plate was embedded in the semi-solid agar by means of sterile forceps. After inoculation with a bacterial suspension, it was kept for 8 days at room temperature. The gummy growth on the porous plate was scraped off, the plate rinsed with distilled water and the polysaccharide obtained as in the usual way. Yield, 0·93 g./l. of medium; ash, 16·8%; N, 5·75%.

*Kieselguhr method.* Nutrient medium (25 ml.), containing the same contents as the usual lucerne root-agar medium with the exception of agar, was poured into each of eight conical flasks. Acid-free kieselguhr (25 g.) was stirred in until a thick paste was obtained. The flasks were sterilized on 3 successive days and then inoculated as above and kept at room temperature for 10 days. Distilled water (40 ml.) was added, the flasks warmed for 3 hr. at 40° and the mixture filtered and carefully washed out with warm distilled water. The combined filtrates were concentrated *in vacuo* at 40–50° and the resultant syrup run into ethanol. Yield, 1·9 g./l. of medium; ash, 34·5%; N, 3·8%.

*Large-scale production of polysaccharide by the Clover Bartel A strain*

As had been mentioned above, strain Clover Bartel A was chosen for large-scale production. It remained active as gum-producer over a very long period and was subcultured every fortnight on slopes of the lucerne-agar metabolism medium. The gum which was obtained on precipitation from ethanol was purified by redissolving it in water and filtering the very dilute solution through kieselguhr. Final ash content, 38·4%.

Purified polysaccharide (60 g.) was obtained, and 200 g. of unpurified material with ash, 68·7%. It was decided not to purify the latter, since it was found that the impurities were separated during a process of methylation and dialysis, which will be described elsewhere.

## SUMMARY

1. Various strains of *Rhizobium radicicolum* species were examined with regard to their ability to produce polysaccharides. Strain Clover Bartel A was found to be the most efficient for large-scale production.

2. Experiments on methods for elimination of agar impurities showed that the use of cellophan was the most satisfactory with regard to yield and purity of the polysaccharide formed.

The authors thank Prof. W. N. Haworth, F.R.S., for his interest in this work and Dr Hugh Nicol for providing cultures.

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# The '*Q*' Notation for Expressing Metabolic Activities in Tissue Slices. A Critical Study

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Ever since Warburg introduced the $Q_{O_2}$ in experiments with tissue slices it has been widely used as a convenient term for comparing experiments performed under different conditions. By expressing the quantity of any substance formed or consumed during a reaction in terms of

$$Q = \frac{\mu\text{l. substance (formed or consumed)}}{\text{mg. tissue (dry wt.)} \times \text{hours of incubation}},$$

it was thought possible to obtain a comparable index of the activity of the tissue even when the conditions of the experiments differed with respect to the amount of tissue used and the period of incubation. While '*Q*' was originally designed to express gaseous changes it has become a general habit of workers to use the term also for changes of non-gaseous substances by assuming the compound formed or consumed to be a gas, of which

1 g. mol. = 22·4 l. In this way besides $Q_{O_2}$, the terms $Q_{\text{amino-N}}$, $Q_{NH_3}$, $Q_{\text{urea}}$, etc., were introduced.

This may be justifiable so long as the weights of tissue and times of incubation are not very different in the experiments to be compared. If, however, they vary widely the use of $Q$ or even the expression of the result per time unit or per unit weight of dry tissue will lead to very considerable errors. It will be seen from the experiments below that no comparable data can be obtained by multiplying, for example, the result of one experiment by a factor $x$ in order to compare it with the result of another experiment in which the amount of tissue used or the time of incubation was $x$ times greater—a procedure frequently adopted. In view of the importance attributed to the $Q$ of a reaction by some workers on problems of intermediary metabolism, a quantitative study of the possible errors involved and an attempt to readjust such erroneous conclusions seemed desirable.

The arginase present in liver slices served as a model in the experiments below, although the conclusions reached may also apply in principle to any work with tissue slices.

## METHODS

*Animals.* Young rats were used, with an average weight of 150 g., fed on ordinary stock laboratory diet. They were killed and the liver was removed as quickly as possible.

*Slices.* The livers were sliced as uniformly as possible and weighed (wet weight). This procedure will be referred to later in detail.

*Incubation of slices.* The method described earlier (Bach, 1939) was closely followed. The total volume of the fluid in the Barcroft vessels varied between 3 and 6 ml. The substrate was added by tipping it from a side-bulb at suitable intervals after the equilibration of the system. At the end of the period of incubation a measured portion of the experimental fluid was taken, acidified with trichloroacetic acid (1% final conc.) and centrifuged. The neutralized supernatant fluid was used for the urea estimation.

*Estimation of urea.* This was carried out by incubation with urease, followed by ammonia distillation according to Parnas & Heller (1924) in the earlier experiments and with the Pregl apparatus modified by Markham (1942) in the later estimations. In many cases additional estimations were carried out by using the manometric method of Krebs & Henseleit (1932).

*Reagents.* $l(+)$-Arginine hydrochloride was prepared from gelatin by the method of Whitmore (1932).

## EXPERIMENTAL

The comparability of two results of experiments with tissue slices expressed as $Q$ (see above), depends (*a*) on the linear relationship between time of incubation and action of the tissue, (*b*) on the linear relationship between quantity of tissue used and its action, (*c*) on the assumption that the dry weight of the tissue is independent of the time of incubation so that tissue slices of identical wet weight will have the same dry weight after different times of incubation.

From the experiments shown in Figs. 1–3 it will be seen that none of these three assumptions applies if times of incubation and quantities of tissue are very different in the experiments to be compared, and the examples given below clearly demonstrate that the $Q$'s of two such reactions are far from comparable.

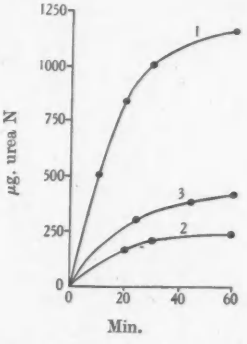


Fig. 1. Hydrolysis of arginine by liver slices. Progress curves with various enzyme and substrate concentrations. Curve 1, 50 mg. slices (wet wt.), 0·5% arginine HCl. Curve 2, 50 mg. slices (wet wt.), 0·075% arginine HCl. Curve 3, 200 mg. slices (wet wt.), 0·075% arginine HCl. Ringer's solution with bicarbonate, total volume 6 ml.

*Comment on Fig.* 1. The three curves represent three incubations of liver slices with arginine over a period of 60 min. Two experiments (2 and 3) differ in the amount of tissue used and two others (1 and 2) in the substrate concentrations. All three experiments show a similar course, i.e. almost complete linearity in the first 10–20 min. and a strong deviation from linearity when incubated for a longer period. Since sufficient substrate was added this deviation could not have been due to exhaustion of the substrate.

It will be seen that the quotient

$$K_1 = \frac{\text{urea-N production after 60 min.}}{\text{urea-N production after 30 min.}}$$

for all three experiments lies between 1·1 and 1·2, whereas it should be 2 if there were a linear relationship.

*Comment on Fig.* 2. The four curves represent four series of incubations of liver slices with arginine for four different periods and two different substrate concentrations. In each series several different portions of slices of varying weight were incubated for the same length of time. Thus the curves show urea production as a function of the quantity of slices incubated, for different periods of incubation. It

will be seen that while urea production approaches proportionality to the amount of tissue used with very short incubation periods, it is far from it with

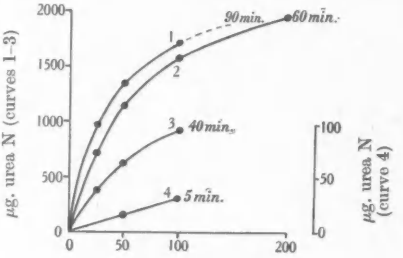


Fig. 2. Hydrolysis of arginine by various quantities of liver slices. Curves 1 and 2, 0·5% arginine HCl. Curves 3 and 4, 0·15% arginine HCl. Ringer solution with bicarbonate (total volume 6 ml.).

60 and 90 min. periods, i.e. with incubation times widely used in experiments on intermediary metabolism. Thus

$$K_2 = \frac{\text{urea production with 100 mg. slices (wet wt.)}}{\text{urea production with 50 mg. slices (wet wt.)}}$$

= 1·8, 1·5, 1·34, 1·26 for periods of 5, 40, 60 and 90 min., respectively.

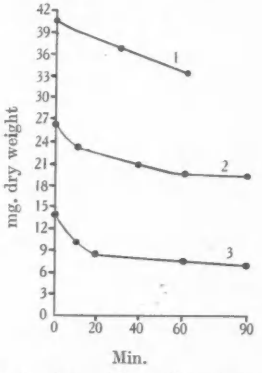


Fig. 3. Loss of weight of liver slices during incubation. Incubation at 37° in Ringer's solution containing bicarbonate, in presence of arginine. Curve 1, 200 mg. Curve 2, 100 mg. Curve 3, 50 mg. wet wt.

*Comment on Fig.* 3. The three curves show the loss in weight of various quantities of liver slices during incubation. A considerable loss is to be expected owing to the diffusion of proteins and other constituents out of the tissue (Elliot, Greig & Benoy, 1937); that such a diffusion occurs is easy to demonstrate by precipitating the proteins in the fluid after the period of incubation. But that the loss of weight can be as large as 50% for 50 mg. slices after 60 min. incubation is surprising. There is a smaller proportional loss with larger quantities of slices.

Thus by taking the dry weights as a basis in the comparison of experimental data a considerable additional error is introduced if the times of incubation vary. Even if the slices were of equal weight before the experiment their dry weights would be different after the incubations.

### *Examples*

The following examples may illustrate the order of magnitude of the errors introduced if the three points mentioned above are left out of consideration.

*First example*

The following experimental data are to be compared:

(*a*) 50 mg. liver slices (wet wt.) were incubated with their substrates for 30 min. and produced 1000 μg. urea-N (Fig. 1, curve 1) = 800 μl.

(*b*) 100 mg. liver slices after incubation for 60 min. gave rise to 1600 μg. urea-N (Fig. 2, curve 2) = 1280 μl.

Dry wts. for (*a*) and (*b*) taken from Fig. 3: 50 mg. wet wt. after 30 min. give 8 mg. dry wt. and 100 mg. wet wt. after 60 min. give 19·5 mg. dry wt.

The orthodox calculation gives for

$$Q = \frac{\mu\text{l.}}{\text{mg. dry wt.} \times \text{hours}}$$

$$Q_a = \frac{800}{8 \times 0{\cdot}5} = 200 \quad \text{and} \quad Q_b = \frac{1280}{19{\cdot}5 \times 1} = 65{\cdot}6.$$

$$\therefore \quad Q_a : Q_b = \text{approx. } 3 : 1.$$

If, however, the conclusions drawn from Figs. 1 and 2 are allowed for, and if the wet weight is taken in place of the dry weight to avoid the errors illustrated in Fig. 3, the following calculation gives the true comparison of the two experiments:

$$\frac{\text{50 mg. tissue incubated 60 min.}}{\text{50 mg. tissue incubated 30 min.}} = \frac{1160}{1020} = \frac{1{\cdot}14}{1}$$

(according to Fig. 1, curve 1) and

$$\frac{\text{60 min. incubation of 100 mg. tissue}}{\text{60 min. incubation of 50 mg. tissue}} = \frac{1550}{1158} = \frac{1{\cdot}34}{1}$$

(according to Fig. 2, curve 2).

$$\therefore \quad \text{for } Q_a' = \frac{800}{1 \times 1}, \quad Q_b' = \frac{1280}{1{\cdot}14 \times 1{\cdot}34}.$$

$$\therefore \quad Q_a' : Q_b' = 800 : 840 = \text{approx. } 1 : 1.$$

The error of 300% shown above is exceeded when the experimental conditions of the two experiments are still farther apart, as in the next example.

*Second example*

(*a*) As before: 50 mg. incubated 30 min. give rise to 1000 $\mu$g. urea-N = 800 $\mu$l.

(*b*) 200 mg. incubated 60 min. give rise to 2000 $\mu$g. urea-N (Fig. 2, curve 2) = 1600 $\mu$l.

Dry wt. from Fig. 3:

50 mg. after 30 min. = 8 mg.,
200 mg. after 60 min. = 33 mg.

$Q_a$ calculated in the orthodox way $= \frac{800}{8 \times 0{\cdot}5} = 200.$

$Q_b$ calculated in the orthodox way $= \frac{1600}{33 \times 1} = 48{\cdot}5.$

$\therefore \quad Q_a : Q_b =$ approx. 4 : 1.

Allowing for disproportionality (Figs. 1, 2):

$$\frac{\text{50 mg. incubated 60 min.}}{\text{50 mg. incubated 30 min.}} = \frac{1160}{1020} = \frac{1{\cdot}14}{1}$$

(Fig. 1, curve 1) and

$$\frac{\text{60 min. incubation of 200 mg.}}{\text{60 min. incubation of 50 mg.}} = \frac{1992}{1158} = \frac{1{\cdot}72}{1}$$

(Fig. 2, curve 2).

$$\therefore \quad Q_a' : Q_b' = \frac{800}{1 \times 1} : \frac{1600}{1{\cdot}14 \times 1{\cdot}72}$$

$$= 800 : 816 = \text{approx. } 1 : 1.$$

*Slicing and weighing of the tissue*

If the weighing is done within 2–3 min. after slicing, the loss of water during weighing is negligible. Blotting the slices before weighing does not improve the accuracy. Table 1 shows a good agreement of wet weight and dry weight (with different periods of incubation) in several parallel weighings.

Table 1. *Reproducibility in the weights of slices*

| Exp. no. | Wet wt. (mg.) | Dry wt. (mg.) | Period of incubation (min.) |
|---|---|---|---|
| I | 50 | 8·8 | 20 |
| I | 50 | 8·8 | 20 |
| | 50 | 9·06 | 20 |
| I | 100 | 25·4 | 0 |
| II | 100 | 24·5 | 0 |
| I | 200 | 34·07 | 60 |
| III | 200 | 33·74 | 60 |
| | 200 | 34·07 | 60 |

## DISCUSSION

Errors of 300–400 % as calculated above are admittedly extreme examples of an erroneous application of the term $Q$. The errors will be less if the $Q$ of experiments with low tissue weight and a long period of incubation are compared with the $Q$ of experiments with high tissue weight and a short period of incubation, because the errors may partially cancel out. It follows, however, that for a fair comparison of two tissue slice experiments one of three conditions must be fulfilled: *either* the times of incubation have to be kept below 30 min. and the weight of tissue below 50 mg. (wet weight) so that small variations in both time and tissue weight are linear; *or* if experimental conditions vary more widely, adjustments have to be made as shown above, based on preceding experimental work similar to that represented in Figs. 1 and 2. For while Fig. 3 may be generally applicable to tissue slices, the course of reactions as shown in Figs. 1 and 2 will probably have to be worked out for every particular case; *or* finally, equal wet weights of tissue and equal times of incubation have to be used in all comparative experiments.

The first requirement will be difficult to meet if slow reactions are involved, such as *in vitro* synthesis, necessitating long periods of incubation and comparatively large amounts of tissue in order to obtain significant effects. The second requirement involves laborious experiments in order to find the relations between time of incubation, weight of tissue, and formation of the products of reaction. But it should be possible to meet the third requirement and to use equal weights and times of incubation throughout the whole series of experiments, though the slicing and the weighing of the moist tissue will need special attention.

## SUMMARY

1. It has been shown that the arginase activity of liver tissue is not proportional to the weight of tissue slices and the period of their incubation (if exceeding 20–30 min.) and that the dry weight of tissue slices decreases with the period of incubation.

2. Both findings contribute to cause considerable errors, amounting to more than 400 % in extreme cases if

$$Q = \frac{\text{metabolic output}}{\text{mg. dry wt.} \times \text{hours of incubation}}$$

is used for the comparison of results from experiments with tissue slices under widely varying experimental conditions. It is suggested that the wet weight rather than the dry weight should be used and the time of incubation and weight of tissue kept constant. The accuracy of slicing and weighing of tissue is discussed.

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# Combination of Potato Virus *X* and Tobacco Mosaic Virus with Pepsin and Trypsin

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It is known that trypsin and pepsin combine with their substrates before causing hydrolysis (Bayliss, 1906; Northrop, 1920), but there is no evidence to show whether such combination is specific in the sense that it is restricted to substrates. Pepsin does not combine with starch, agar, kaolin or $CaSO_4$ (Northrop, 1920), but these differ so widely from proteins that this fact cannot be interpreted as indicating that substrates have a specific affinity for enzymes.

The two plant viruses, potato virus *X* and tobacco mosaic virus, seemed to afford particularly suitable material for studying this problem, for they are both nucleoproteins with rod-shaped particles and with many similar properties, but potato virus *X* is readily hydrolyzed by both pepsin and trypsin (Bawden & Pirie, 1936, 1938), whereas tobacco mosaic virus is not. However, denaturation by heat renders tobacco mosaic virus susceptible to proteolysis by both enzymes. Both viruses are readily obtained in large quantities and in the form in which they give liquid crystalline solutions. Their large size and insolubility around pH 4·0 are properties that permit easy separation from dissolved free enzymes, and their specific activities provide additional tests for changes too small to be detected by the chemical and physical tests used for studying most proteins.

Previous work has shown that although trypsin does not hydrolyze tobacco mosaic virus, mixing the enzyme with the virus leads to an immediate loss of infectivity (Caldwell, 1933; Stanley, 1934*a*; Bawden & Pirie, 1937); this phenomenon is reversible and fully infective virus can be recovered from such mixtures. Mixing trypsin with potato virus *X* also leads to an immediate drop in infectivity, but, at pH values at which trypsin is proteolytically active, this is followed by hydrolysis (Bawden & Pirie, 1936).

The action of pepsin on tobacco mosaic virus has been a subject of some controversy. The addition of the enzyme to the virus has no immediate effect, but Stanley (1934*b*) and Ross & Vinson (1937) claimed that after incubation for many days at pH 3·0 and 37° the virus lost infectivity, whereas Bawden & Pirie (1937), working with liquid crystalline virus preparations, found that pepsin had no effect, although it rapidly hydrolyzed the coagulum produced by heat denaturation of the virus. Experiments made in the course of the present work confirm Bawden & Pirie, for the virus preparations used were unaffected by as much as 10 days' incubation with 0·1% pepsin at pH 3·0 and 37°.

In this work the ability of pepsin, trypsin and invertase to combine with the two viruses was tested, and certain effects of adsorbing the three enzymes by charcoal were also studied. Invertase was included to compare the behaviour of a non-proteolytic enzyme, which has no effect on the two viruses or on their components, with that of pepsin and trypsin.

## MATERIAL AND METHODS

*Liquid crystalline preparations of the viruses.* Tobacco mosaic virus was isolated from sap expressed from minced leaves of infected tomato plants by the method described by Bawden & Pirie (1943), involving several successive precipitations by one-third saturation with $(NH_4)_2SO_4$, and with dilute HCl at pH 3·3.

Potato virus *X* was isolated from sap expressed from minced leaves of infected tobacco plants by a method similar to that described by Bawden & Pirie (1938). The sap was frozen overnight. After several successive precipitations by one-quarter saturation with $(NH_4)_2SO_4$ and with dilute HCl at pH 4·5 the preparations were dialyzed; the virus was then sedimented by centrifugation for 1 hr. at 40,000 r.p.m. and redissolved in water.

*Enzymes.* The commercial preparations of pepsin and invertase, used in this work, were obtained from The British Drug Houses Ltd. As a source of trypsin E. Merck's 'Pancreatin' was used.

*Methods of testing combination.* Solutions of the enzymes were mixed with solutions or suspensions of substances under test. The mixtures were centrifuged in conditions in which the substances tested for combination with the enzymes, but not the free enzymes, are sedimented. The enzymes remaining in the supernatant fluids were then estimated quantitatively.

*Quantitative estimations of the enzymes.* Pepsin and trypsin were incubated, respectively, with acid or slightly alkaline casein solutions for a given period at 37° and the casein which still remained precipitable by 1% trichloroacetic acid was estimated gravimetrically, acid solutions being neutralized before the addition of trichloroacetic acid.

Invertase was incubated with sucrose for a given period at room temperature, and the amount of reduction of Fehling's solution by the reducing sugar formed was estimated by a micro-Bertrand method.

The relative concentrations of the enzymes were calculated by comparing their effects with those of different concentrations of control enzyme solutions incubated simultaneously in the same conditions. If the effects increased with concentrations in a non-linear manner, the calculations were made by graphic interpolation, and if they increased linearly, by the ratio of the effect of tested solutions to that of the control.

In the experimental conditions used in this work amounts of casein rendered soluble in 1% trichloroacetic acid by pepsin increased with increasing concentrations of pepsin in a non-linear manner. On the other hand, there was a direct proportionality between amounts of casein rendered soluble in 1% trichloroacetic acid by trypsin and concentrations of trypsin, and also between amounts of reducing sugar formed from sucrose and concentrations of invertase.

*Serological precipitin tests* were made as previously described (Kleczkowski, 1943). Increasing dilutions of virus preparations were titrated against constant dilutions of antisera.

*Infectivity tests* were made by the local lesion method; tobacco mosaic virus was tested on *N. glutinosa*, and potato virus *X* on *N. tabacum* var. White Barley.

## EXPERIMENTAL

### *Experiments with pepsin*

Table 1 gives the results of an experiment to test the ability of pepsin at pH 4·0 to combine with suspensions of the two viruses and with a suspension of heat-denatured tobacco mosaic virus.

The suspensions of tobacco mosaic virus were made by precipitating the virus at pH 3·5, centrifuging down the precipitate and resuspending it in pH 4·0 buffer. Potato virus *X* was precipitated by one-quarter saturation with $(NH_4)_2SO_4$ and resuspended in pH 4·0 buffer. The suspension of heat-denatured tobacco mosaic virus was made by resuspending in pH 4·0 buffer the precipitate produced by heating, at 100°, a virus solution for 5 min. at pH 7·0 in the presence of 1% NaCl.

The suspensions were mixed with a pepsin solution and immediately centrifuged for 5 min. at 10,000 r.p.m., and pepsin was estimated in the supernatant fluids. It will be seen that potato virus *X* and heat-denatured tobacco mosaic virus removed most of the pepsin from the solutions, whereas native tobacco mosaic virus did not remove any detectable quantity.

Similarly there was no detectable combination between tobacco mosaic virus and pepsin at pH 3·0 and 3·5, whereas potato virus *X* combined almost as much pepsin at pH 4·5 as at pH 4·0. The method described could not be used over a wider pH range, as the viruses can be sedimented by low speed centrifugation only over a narrow pH range around their isoelectric points.

As heat-denatured tobacco mosaic virus is insoluble over a wide pH range, its combination with pepsin could be tested over a wide pH range. Fig. 1 (curve *A*) shows that maximum combination was at pH about 2·5, the same value as that found by Northrop (1920) for maximum combination between pepsin and coagulated egg albumin. This does not correspond, however, with maximal hydrolysis of heat-denatured tobacco mosaic virus during 1 hr. incubation at 37° (cf. line *B*, Fig. 1).

Table 1. *Combination of pepsin with unchanged and heat-denatured tobacco mosaic virus, and with potato virus X*

| | Test mixture | | Data for supernatant fluid from centrifugal test mixture* | | |
|---|---|---|---|---|---|
| Test no. | 1 ml. buffer (pH 4·0) containing virus (mg.) | 1 ml. $H_2O$ containing pepsin (%) | Precipitate obtained with trichloroacetic acid (mg.) | Casein digested† (mg.) | Pepsin removed by virus‡ (% of total) |
| | | (*a*) *Unchanged tobacco mosaic virus* | | | |
| 1 | 4 | 0·1 | 8·1 | 15·4 | 0 |
| 2 | 2 | 0·1 | 8·3 | 15·2 | 0 |
| 3 | 1 | 0·1 | 8·0 | 15·5 | 0 |
| | | (*b*) *Denatured tobacco mosaic virus* | | | |
| 4 | 4 | 0·1 | 15·9 | 7·6 | 80 |
| 5 | 2 | 0·1 | 10·4 | 13·1 | 50 |
| 6 | 1 | 0·1 | 9·1 | 14·4 | 25 |
| | | (*c*) *Potato virus X* | | | |
| 7 | 4 | 0·1 | 19·0 | 4·5 | 90 |
| 8 | 2 | 0·1 | 17·8 | 5·7 | 85 |
| 9 | 1 | 0·1 | 13·0 | 10·5 | 65 |
| | | (*d*) *Control solutions—no virus* | | | |
| 10 | — | 0·1 | 8·2 | 15·3 | — |
| 11 | — | 0·05 | 10·4 | 13·1 | — |
| 12 | — | 0·025 | 15·5 | 8·0 | — |
| 13 | — | 0·012 | 17·0 | 6·5 | — |
| 14 | — | 0·006 | 20·2 | 3·3 | — |
| 15 | — | 0·0 | 23·5 | — | — |

* The mixtures of the viruses with pepsin were immediately centrifuged for 5 min. at 10,000 r.p.m. and supernatant fluids decanted. To 1 ml. of each fluid 2 ml. of 1·2% acid casein were added and the mixtures were incubated for 3 hr. at 37°; they were then neutralized and 0·3 ml. of 10% trichloroacetic acid added to each mixture. The precipitates were centrifuged down, resuspended in 2 ml. $H_2O$, filtered off, dried and weighed.

† The differences between 23·5 mg. and the amounts precipitated by trichloroacetic acid.

‡ Computed approximately by graphic interpolation from the data of the tests nos. 10–15.

The ability of pepsin to combine with casein, heat-denatured human serum globulin, insoluble starch, kaolin and charcoal was also tested by the same method. The results, given in Table 2, show that casein, globulin and charcoal combined with most of the pepsin, but there was no detectable combination with starch and kaolin.

To see whether pepsin could combine with potato virus *X* without causing any detectable changes, the precipitate of the virus centrifuged from the mixture with pepsin (test no. 7, Table 1) was immediately redissolved in 2 ml. of M/15 phosphate buffer

at pH 7·0. The general appearance of the solution and of its anisotropy of flow, the nitrogen content (micro-Kjeldahl), its serological precipitin titre and its infectivity at 1/1000, did not differ from those of the control preparation. The precipitate of the heat-denatured tobacco mosaic virus, centrifuged from the mixture with pepsin (test no. 4, Table 1), also contained the same amount of nitrogen as a control suspension. Thus it seems that combination of pepsin with potato virus *X* or with heat-denatured tobacco mosaic virus takes place before any further action of the enzyme upon the two substrates becomes noticeable.

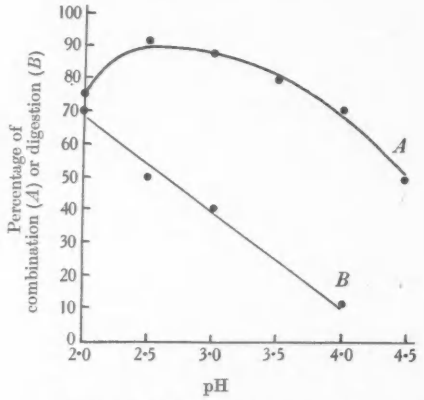


Fig. 1. The effect of pH on combination of pepsin with heat-denatured tobacco mosaic virus and on the rate of digestion during 1 hr. Curve *A*, percentage of pepsin combined. Curve *B*, percentage of virus protein dissolved by pepsin. *Combination.* 1 ml. of buffer solutions of different pH's containing 2 mg. of the denatured virus was mixed with 1 ml. of 0·1% pepsin. Virus suspensions were removed immediately by a short centrifugation, all the supernatants adjusted to the same pH (3·0) and the amounts of pepsin estimated as described in Table 1. *Rate of digestion.* 1·0% suspensions of the denatured virus in buffers of different pH's were incubated for 1 hr. at 37° with 0·15% pepsin. Then the suspensions were centrifuged down and the nitrogen estimated (micro-Kjeldahl).

Table 2. *Combination of pepsin with various substances*

1 ml. of buffer solutions containing suspensions of given substances was mixed with 1 ml. of 0·1% pepsin solution and centrifuged for 5 min. at 10,000 r.p.m. immediately, or after a period of incubation, and the amounts of pepsin in the supernatant fluids estimated as described in Table 1.

| Substance tested for combination | | | Period of incubation (min.) | Pepsin removed (approx. % of total) |
|---|---|---|---|---|
| Name | Amount (mg.) | pH | | |
| Casein | 5·0 | 4·5 | 0 | 90 |
| | 2·5 | 4·5 | 0 | 80 |
| | 1·2 | 4·5 | 0 | 70 |
| | 0·6 | 4·5 | 0 | 50 |
| Denatured human serum globulin | 2·5 | 4·0 | 20 | 95 |
| Charcoal | 5·0 | 4·0 | 20 | 85 |
| Kaolin | 5·0 | 4·0 | 20 | 0 |
| Starch | 5·0 | 4·0 | 20 | 0 |

When a 0·2% suspension of potato virus *X* at pH 4·0 was incubated with 0·05% pepsin for 12 hr. at 37°, the fluid became water-clear and a small resinous mass (nuclein) floated at the surface. Anisotropy of flow, serological activity and infectivity were lost. The fluid did not give a precipitate on one-quarter saturation with $(NH_4)_2SO_4$ or with 1% trichloroacetic acid. A control incubated in the same conditions without pepsin remained apparently unchanged. The precipitate of heat-denatured tobacco mosaic virus, incubated similarly with pepsin for 12 hr., was entirely dissolved, and the resulting water-clear solution did not give a precipitate on one-third saturation with $(NH_4)_2SO_4$ or (after neutralization) with 1% trichloroacetic acid. A similarly treated control incubated without pepsin remained apparently unchanged.

For comparison a 0·2% suspension of native tobacco mosaic virus, at pH 3·0, was incubated with 0·1% pepsin for 10 days at 37°, and then centrifuged down and redissolved in M/15 phosphate buffer at pH 7·0. The general appearance of the solution and of its anisotropy of flow did not differ from those of a control incubated in similar conditions without pepsin, neither was there any difference in the amount of nitrogen, in the serological titre or in infectivity (inoculated at a dilution of 1/1000 into 18 half-leaves of *N. glutinosa* it gave 43 lesions per leaf compared with 39 given by the control).

*Experiments with trypsin*

To test the ability of trypsin to combine with tobacco mosaic virus and with potato virus *X* at pH 7·0, high-speed centrifugation was used. The viruses were sedimented from mixtures with the enzyme by centrifugation for 1 hr. at 40,000 r.p.m., and the trypsin remaining in the supernatant fluids was then estimated. Table 3 gives the details and results of the experiment; each virus removed some trypsin, but tobacco mosaic virus removed more than potato virus *X*, and the difference became greater with increasing concentration of trypsin.

In tests with pepsin and potato virus *X* at pH 4·0, the virus could be removed from mixtures before undergoing any detectable changes. This could not be done with mixtures of potato virus *X* and trypsin at pH 7·0, as centrifugation necessitated 2½ hr. exposure of the virus to trypsin (1 hr. at the top speed). The pellets of potato virus *X* sedimented from the mixtures with trypsin (tests nos. 1 and 5, Table 3)

Table 3. *Combination of trypsin with potato virus X and with tobacco mosaic virus at* pH 7·0

0·1 and 0·025% solutions of trypsin in water (pH 7·0) containing 0·1% of potato virus *X* or tobacco mosaic virus, and controls without the viruses, were centrifuged at 40,000 r.p.m., so that both viruses were sedimented. Trypsin was then estimated in the supernatant fluids. 1 ml. of each supernatant fluid was added to 2 ml. of 1% casein solution (pH 7·3). The mixtures were incubated for given periods of time at 37°, then 0·3 ml. of 10% trichloroacetic acid was added to each mixture; the precipitates were centrifuged down, resuspended in 2 ml. water, filtered off, dried and weighed.

| Test no. | Material tested for combination | Conc. of trypsin (%) | Time of incubation (hr.) | Casein precipitated by trichloro-acetic acid (mg.) | Casein digested (mg.) | Trypsin removed* (% of total) |
|---|---|---|---|---|---|---|
| 1 | Potato virus *X* | 0·1 | 3 | 9·8 | 10·7 | 5 |
| 2 | Tobacco mosaic virus | 0·1 | 3 | 12·9 | 7·6 | 30 |
| 3 | Trypsin control | 0·1 | 3 | 9·2 | 11·3 | — |
| 4 | None | 0·0 | 3 | 20·5 | — | — |
| 5 | Potato virus *X* | 0·025 | 6 | 16·0 | 4·5 | 25 |
| 6 | Tobacco mosaic virus | 0·025 | 6 | 16·7 | 3·8 | 40 |
| 7 | Trypsin control | 0·025 | 6 | 14·6 | 5·9 | — |
| 8 | None | 0·00 | 6 | 20·5 | — | — |

* As within the tested range of trypsin concentrations and incubation times the amounts of digested casein were directly proportional to trypsin concentrations, percentages of trypsin removed were directly computed from the ratio of casein digested by tested solutions to that digested by the control.

were immediately redissolved in water, precipitated by one-quarter saturation with $(NH_4)_2SO_4$ and redissolved in the original volume of water. Although the general appearance and anisotropy of flow of the preparations did not differ noticeably from those of the control, their infectivity and serological activity were reduced (Table 4).

Although trypsin cannot hydrolyze tobacco mosaic virus, it does combine with the virus, and this offers a possible explanation of reversible inhibition of infectivity. There was too much trypsin in the pellets sedimented from mixtures of the enzyme with tobacco mosaic virus (Table 3, tests nos. 2 and 6) for it to be contained as a solution in the free water of the pellets. This could be seen from the fact that the volume of the pellets was only a small fraction of that of fluids from which they

Table 4. *Infectivity and serological activity of potato virus X sedimented by high-speed centrifugation from its mixture with trypsin*

| Tested material | Serological titre | Infectivity. Average number of lesions/leaf with material diluted 1/100 | 1/1000 |
|---|---|---|---|
| Pellet from 0·1% trypsin solution (test no. 1, Table 3) | 1/320 | 48 | 10 |
| Pellet from 0·025% trypsin solution (test no. 5, Table 3) | 1/480 | 51 | 11 |
| Control | 1/640 | 63 | 17 |

were sedimented. Hence most of the trypsin must have been combined with the virus. The experiment shown in Table 5 was made to see how easily this combination could be broken by washing with water. A pellet sedimented by a high-speed centrifugation from a mixture of the virus and trypsin, at pH 7·0, was redissolved in water and the solution was centrifuged again. It will be seen that about one-half of the trypsin was thus extracted from the virus by water, which suggests that the combination is readily reversible.

Table 5. *Removal of trypsin from tobacco mosaic virus by washing at* pH 7·0

15 ml. of a mixture containing 0·05% trypsin and 0·2% tobacco mosaic virus were centrifuged for 1 hr. at 40,000 r.p.m. The pellet was dissolved in water added to make up the volume to 7·5 ml. Half was kept as redissolved *first pellet*, the other half was made up to 7·5 ml. with water, again centrifuged and the pellet dissolved in water added to make up the volume to 3·75 ml. This was described as the dissolved *second pellet*.

Solutions of each pellet, and also solutions of the virus not treated with trypsin, were mixed with double volumes of water or 1·25% casein at pH 7·3. The mixtures were incubated for 6 hr. at 37°, then 0·3 ml. of 10% trichloroacetic acid was added, the precipitates were centrifuged down, resuspended in 2 ml. water, filtered off, dried and weighed.

| Material tested for tryptic activity. 1 ml. of solution of | Water added (ml.) | Casein solution added (1 ml. of 1·25%) | Precipitate with trichloro-acetic acid (mg.) | Casein digested (mg.) |
|---|---|---|---|---|
| First pellet | 0 | 2 | 19·0 | 10·0 |
| | 2 | 0 | 3·7 | — |
| Second pellet | 0 | 2 | 23·5 | 5·5 |
| | 2 | 0 | 3·8 | — |
| 0·4% tobacco mosaic virus | 0 | 2 | 28·9 | 0 |
| | 2 | 0 | 4·0 | — |
| None | 1 | 2 | 25·1 | — |

The ability of trypsin to combine with heat-denatured tobacco mosaic virus at pH 4·0 and 7·0 could be tested by low-speed centrifugation. Tobacco

mosaic virus was denatured by heating for 5 min. at 100° in the presence of 0·5 % NaCl, centrifuged down and resuspended in buffer solutions of the required pH. Equal volumes of 0·1 % trypsin were added, the mixtures were immediately centrifuged, and the trypsin in the supernatant fluids was estimated in the usual way. The results, given in Table 6, show that a proportion of trypsin was combined at both pH values, but considerably more at pH 7·0 than at pH 4·0.

Table 6. *Combination of heat-denatured tobacco mosaic virus with trypsin at* pH 4·0 *and* 7·0

| Concentration of virus suspension mixed with trypsin (%) | Percentage of trypsin removed | |
|---|---|---|
| | At pH 4·0 | At pH 7·0 |
| 0·4 | 20 | 70 |
| 0·2 | 10 | 60 |
| 0·1 | 5 | 55 |

The ability of trypsin to combine with suspensions of tobacco mosaic virus and potato virus *X* at pH 4·0 was also tested, and compared with the ability to combine with heat-denatured tobacco mosaic virus. The mixtures contained 0·2 % virus suspensions and 0·015 % trypsin. Heat-denatured tobacco mosaic virus combined with about 25 % and native tobacco mosaic virus with slightly less trypsin, whereas potato virus *X* did not combine with any amount detectable by this method.

*Experiments with invertase*

In a further attempt to assess the specificity of combination of pepsin and trypsin with their substrates, the ability of invertase to combine with the two viruses and with casein was tested. To ensure that combination with amounts of invertase comparable to those of pepsin or trypsin should not be missed, it was desirable that the concentration of invertase used should be the same or smaller than the concentration of pepsin and trypsin. It is believed that this condition was fulfilled by using 0·002 % solution of the invertase preparation for comparison with 0·05 % solutions of pepsin and trypsin, for at these concentrations comparable proportions of the three enzymes were adsorbed by 0·5 % charcoal.

1 ml. of 0·004 % invertase was mixed with 1 ml. of 1 % suspensions of each of the following substances: tobacco mosaic virus in pH 3·5 buffer, heat-denatured tobacco mosaic virus in pH 3·5 buffer, potato virus *X* in pH 4·0 buffer and casein in pH 4·5 buffer. After 90 min. at room temperature the suspensions were centrifuged and invertase estimated in the supernatant fluids. 0·25 ml. of 5 % sucrose solution was added to 1 ml. of each supernatant fluid and after 4 hr. incubation at room temperature the amounts of hydrolyzed sucrose were estimated. In these conditions the amounts of reducing sugar were found to be directly proportional to invertase concentration, so that invertase in the tested solutions could be computed from the ratio of the amount of reducing sugar to that in the control solutions of invertase.

All the supernatant fluids contained the same amounts of invertase as their respective controls, showing that none of the protein suspensions combined with any detectable amount of invertase. In similar conditions 1 % charcoal suspension in pH 4·0 buffer removed 90 % of the invertase.

The ability of invertase to combine with tobacco mosaic virus in solution was also tested at pH 6·5. 0·004 % invertase solution in pH 6·5 M/15 phosphate buffer was mixed with an equal volume of 1 % solution of the virus at the same pH. The virus was then sedimented by centrifugation for 1 hr. at 40,000 r.p.m., and invertase estimated in the supernatant fluid. The supernatant fluid had the same invertase activity as a control solution of invertase not mixed with the virus but otherwise treated similarly. Thus no detectable amount of invertase combined with the virus.

The result of the experiment was checked by testing the pellet sedimented from the mixture of the virus and invertase for the presence of invertase. The pellet was redissolved in the original volume of pH 6·5 buffer, and 2 ml. of the solution was mixed with 0·5 ml. of 5 % sucrose. After incubation for 3 hr. at room temperature the virus was removed from the mixture by adding 0·2 ml. of $\frac{2}{3}$ N-$H_2SO_4$ and 0·2 ml. of 10 % sodium tungstate. The precipitate was centrifuged off and the supernatant fluid was tested for reducing sugar. None was detected. To exclude the possibility that the presence of the virus might have interfered with enzymic activity of invertase, 2 ml. of 0·5 % solution of the virus in pH 6·5 buffer, containing 0·002 % invertase, was also mixed with 0·5 ml. of 5 % sucrose, incubated simultaneously and then tested similarly. It contained the same amount of hydrolyzed sucrose as a control solution containing only invertase and sucrose.

*Some effects of adsorption of the enzymes by charcoal. Extraction of the enzymes from charcoal. Protection of trypsin by tobacco mosaic virus from spontaneous inactivation at* pH 7·0

Hedin (1906) found that the addition of sufficient charcoal to adsorb almost all the trypsin in a solution reduced its enzymic activity, and that this reduction of activity increased with the length of time the charcoal and the enzyme were in contact before the substrate (casein) was added. That most of the trypsin was adsorbed could be shown by filtering

off the charcoal before adding casein, when the filtrate showed almost no tryptic activity. Table 7 shows that the activity of trypsin adsorbed on charcoal steadily decreased during the period between adding charcoal and casein. This loss of activity is probably due to denaturation of trypsin on the surface of the charcoal.

Table 7. *The effect of incubating trypsin with charcoal at* pH 7·0

1 vol. of 1·5% water-suspension of charcoal was added to 2 vol. of 0·06% trypsin solution. The mixture was incubated for given lengths of time at room temperature and then 6 vol. of 1% casein (pH 7·0) were added, and the mixture incubated for 3 hr. at 37°. In one group of experiments charcoal was removed (by centrifugation) before casein was added, and in the other it was not removed. The amounts of digested casein were then estimated as in Table 3. Charcoal was removed immediately before the estimation.

| Time of incubation of trypsin-charcoal mixture (min.) | Treatment | Casein digested (mg. in 3 ml. of mixture) | Tryptic activity remaining (% of total)* |
|---|---|---|---|
| 0 | Charcoal removed before casein was added | 2·2 | 21 |
| 5 | | 2·0 | 19 |
| 15 | | 1·8 | 17 |
| 60 | | 1·2 | 11 |
| 0 | Charcoal not removed | 8·8 | 84 |
| 5 | | 6·7 | 64 |
| 15 | | 5·1 | 49 |
| 60 | | 2·0 | 19 |
| — | Control (water added instead of charcoal suspension) | 10·5 | — |

* Computed from the ratio of casein digested by a tested sample to that digested by the control (see Table 3).

A similar experiment with pepsin (with the same concentrations of the reagents in buffer at pH 4·0) showed that the activity of pepsin adsorbed on charcoal also steadily decreased during the period between adding charcoal and casein, but the rate of the decrease was much slower than with trypsin. The fall in the activity after 24 hr. was approximately equal to that of trypsin after 30 min.

Although tobacco mosaic virus combines with trypsin, the presence of the virus has no effect on the ability of the enzyme to hydrolyze casein. Indeed, the presence of the virus protects trypsin from the spontaneous inactivation which it normally undergoes during incubation at pH 7·0. This protective effect of the virus is shown by the experiment described in Table 8. One-half of the tryptic activity of a trypsin solution was destroyed during 24 hr. at 37°, whereas only one-fifth was lost in the presence of the virus.

Table 8. *Protection of trypsin by tobacco mosaic virus*

0·1% trypsin solution was mixed with an equal volume of 1% tobacco mosaic virus or with water and the mixtures incubated for 24 hr. at 37°. Then 4 vol. of 1% casein solution (pH 7·0) were added to each mixture and the mixtures were incubated for 4 hr. at 37°. Amounts of digested casein were then estimated as described in Table 3.

| Treatment of trypsin before incubation with casein | Casein digested (mg. in 3 ml. of mixture) | Tryptic activity remaining (% of total) |
|---|---|---|
| Mixture trypsin + virus incubated for 24 hr. at 37° | 15·25 | 80 |
| Trypsin and virus incubated separately for 24 hr. at 37° and then mixed | 9·5 | 50 |
| Trypsin + water incubated for 24 hr. at 37° | 9·8 | 52 |
| Trypsin freshly dissolved | 18·9 | 100 |

Table 9. *Extraction of trypsin from charcoal by casein*

0·15% trypsin solutions were mixed with equal volumes of 1·5% charcoal suspensions in water or with equal volumes of water (control). 4 vol. of 1% casein were added, and the mixtures were incubated at 37°. Charcoal was removed by centrifugation at given times. Amounts of digested casein were estimated as described in Table 3 after 1 hr. and after 3 hr. incubation.

| Test no. | Time at which charcoal was removed | Casein digested (mg. in 3 ml. of mixture) after 1 hr. | Casein digested (mg. in 3 ml. of mixture) after 3 hr. |
|---|---|---|---|
| 1 | Before adding casein | — | 2·0 |
| 2 | Immediately after adding casein | — | 6·2 |
| 3 | 1 hr. after adding casein | 4·3 | 10·3 |
| 4 | At the end of incubation with casein | 4·3 | 11·5 |
| 5 | Control (no charcoal added) | 9·4 | 16·0 |

Hedin (1907) showed that casein can set free trypsin adsorbed on charcoal. The experiment described in Table 9 confirms this. It can be seen that the removal of charcoal from a trypsin solution, immediately after adding casein, reduced tryptic activity of the solution much less than if the charcoal was removed before adding casein. After incubation for 1 hr. at 37°, casein liberated most of the trypsin, for removing the charcoal then had little influence on subsequent hydrolysis of casein. Since casein liberated the adsorbed trypsin, it was impossible to determine directly whether trypsin was active while adsorbed on charcoal.

When charcoal was added to a solution of 0·1% trypsin already containing 1% casein, the presence of casein entirely prevented adsorption of the trypsin by charcoal. The presence of 1% tobacco mosaic

virus in 0·1% trypsin could not prevent the adsorption, though it considerably reduced the amount of trypsin adsorbed. However, when the virus was added to a solution of trypsin already containing charcoal, it could not liberate any trypsin already adsorbed on charcoal.

can hydrolyze sucrose, although the rate of hydrolysis was somewhat slower than with free invertase. That the enzyme remained attached to charcoal was shown by the fact that at any time during incubation the hydrolysis could be stopped by removing the charcoal.

Table 10. *Extraction of invertase from charcoal by casein at* pH 6·0

3 ml. of 0·0025% invertase solution in pH 6·0 buffer were mixed with 0·3 ml. of 5% charcoal in the buffer or with 0·3 ml. of the buffer, and the mixtures incubated for 1½ hr. at room temperature. 0·3 ml. of 5% casein in the buffer, or 0·3 ml. of the buffer, were added immediately after charcoal or at the end of the incubation period. At the end of the incubation period enough 10% sucrose was added to give 1% sucrose solution, and the mixtures were incubated for 4 hr. at room temperature. Charcoal was removed by centrifugation at given times. At the end of the incubation 1/10 vol. of ⅔ N-$H_2SO_4$ and of 10% sodium tungstate were added to all solutions, the precipitates of casein were removed by centrifugation, and 1 ml. of each solution was assayed for reducing activity.

| Time at which casein was added | Time at which charcoal was removed | Reducing power of solution (mg. Cu/ml.) |
|---|---|---|
| None added | Immediately before adding sucrose | 0·84 |
| None added | Immediately after adding sucrose | 0·89 |
| Immediately after charcoal | Immediately before adding sucrose | 3·01 |
| 1½ hr. after charcoal | Immediately before adding sucrose | 2·37 |
| None added | At the end of the incubation with sucrose | 2·56 |
| None added | No charcoal added (control) | 3·20 |

Table 11. *Activity of invertase adsorbed on charcoal*

1 vol. of 5% charcoal suspension was added to 9 vol. of 0·002% invertase solution in pH 6·0 buffer, and the mixture incubated at room temperature for 1½ hr. Then enough 12% sucrose was added to give 1% sucrose solution. Charcoal was removed by centrifugation at given times, and 1 ml. of each solution assayed for reducing activity after 1½ and 4 hr. incubation with sucrose.

| Time at which charcoal was removed | Reducing activity (mg. Cu/ml.) after 1½ hr. | Reducing activity (mg. Cu/ml.) after 4 hr. |
|---|---|---|
| Immediately before adding sucrose | 0·06 | 0·16 |
| Immediately after adding sucrose | 0·08 | 0·20 |
| 1½ hr .after adding sucrose | 0·83 | 0·85 |
| At the end of incubation with sucrose | | 2·30 |
| Control (buffer added instead of charcoal suspension) | 1·10 | 2·94 |

The experiment described in Table 10 shows that casein solution at pH 6·0 could also remove invertase from charcoal, whereas sucrose could not (see also Table 11). Human serum albumin and tobacco mosaic virus also extracted invertase from charcoal, although albumin was less effective than casein, and the virus less than albumin. The extraction of an enzyme from charcoal, therefore, is clearly no indication that the substance responsible for the extraction is a substrate or that it can even combine with the enzyme.

Nelson & Griffin (1916) found that invertase adsorbed on charcoal hydrolyzed sucrose at the same rate as did the free enzyme. The experiment described in Table 11 confirms that adsorbed invertase

## DISCUSSION

It is clear from the results described in this paper that no general statement can be made about the specificity of forces leading to combination between proteolytic enzymes and their substrates, for individual enzymes differ in their behaviour. The experiments with pepsin all suggest that there are such forces, as this enzyme combined with all the substrates used but did not combine with tobacco mosaic virus. When this virus was turned into a substrate as a result of denaturation by heat, combination could then take place. Thus details of structure of proteins that determine their susceptibility to proteolytic activity of pepsin also seem to determine the ability of pepsin to combine with proteins. This, however, is not true of trypsin, which combined with all the proteins tested. Indeed, more trypsin entered into combination with tobacco mosaic virus, which is not a substrate for its proteolytic activity, than with potato virus *X*, which is a substrate. This combination does not seem to be simple adsorption of the enzyme by the virus, for tobacco mosaic virus did not combine with pepsin and neither virus combined with invertase. By contrast, charcoal, which presumably combined by surface adsorption, combined with all three enzymes and with all the other proteins used. The liberation of enzymes from charcoal by proteins cannot be considered as evidence of their affinity to the enzymes, for it can also occur with enzymes for which the proteins are not substrates, and with which they do not combine. The liberation is most

likely a result of replacement of one adsorbed protein by another.

The immediate inhibition of infectivity when trypsin is added to solutions of some plant viruses has been interpreted differently by different authors. Stanley (1934*a*) concluded from the results of his infectivity experiments with tobacco mosaic virus that trypsin was affecting the susceptibility of the host plant and not the virus. He found no difference between the rate of diffusion of trypsin in the presence and absence of the virus and took this as evidence that there was no combination. Bawden & Pirie (1936) suggested that the virus and trypsin might combine to give a non-infective complex, which breaks down on dilution; they pointed out that Stanley's diffusion experiments might have been made with such an excess of trypsin that no differences in the diffusion rate could have been detected even if such a complex were formed. Stanley himself took this possibility into account, and, therefore, did not consider his evidence as a proof of lack of combination. Since then, Hills & Vinson (1938) have found that the diffusion rate of both trypsin and the virus are influenced when the two are present in a solution together. The experiments described here do not exclude the possibility that trypsin affects the susceptibility of the host plant. However, they do show that trypsin combines with tobacco mosaic virus and with potato virus *X*, and that the combination is fairly readily broken by dilution in water, and it seems most reasonable that the union of trypsin with the viruses leads to inhibition of infectivity.

A similar effect has been described by Loring (1942) with ribonuclease and tobacco mosaic virus. Ribonuclease, which decomposes nucleic acid separated from the denatured virus, has no effect on the intact virus, but it combines with it and causes an inhibition of infectivity. Trypsin and ribonuclease have two features in common that might account for their ability to combine with tobacco mosaic virus. Both are able to decompose parts of the denatured virus, so that there may be some specific attraction between these parts of the intact virus and the enzymes, even though some structural details prevent enzymic decomposition. Secondly, both enzymes have isoelectric points near pH 7·0, whereas the isoelectric point of the virus is at pH 3·3, so that their combination with the virus could result from differences of charge.

Mere combination of potato virus *X* with pepsin has no effect on infectivity, which suggests that different parts of the virus particle are involved in combination with pepsin and trypsin. As combination between tobacco mosaic virus and trypsin protects trypsin from spontaneous inactivation at pH 7·0 and also causes inhibition of infectivity of the virus, the combination seems to involve parts of the virus essential for infectivity, on the one side, and active parts of the enzyme, on the other side.

## SUMMARY

1. Pepsin combines with potato virus *X* and casein, which are substrates for its proteolytic activity, but not with tobacco mosaic virus, which is not a substrate.

2. Tobacco mosaic virus denatured by heat is readily hydrolyzed by pepsin and combines with pepsin almost to the same extent as potato virus *X*.

3. Invertase does not combine with potato virus *X*, with tobacco mosaic virus, whether heat-denatured or not, or with casein.

4. More trypsin combines with tobacco mosaic virus, which is not a substrate for its proteolytic activity, than with potato virus *X*, which is a substrate. The combination of trypsin with tobacco mosaic virus could account for the reversible inhibition of infectivity of the virus by trypsin.

5. Combination between trypsin and tobacco mosaic virus protects trypsin from spontaneous inactivation at pH 7·0.

6. Trypsin and invertase adsorbed on charcoal can be set free by casein; invertase can also be extracted by tobacco mosaic virus, but not by sucrose.

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# The Nature of the *Aeromonas* Fermentation

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The genus *Aeromonas* was established by Kluyver & van Niel (1936) for pseudomonads fermenting carbohydrates with the production of carbon dioxide and hydrogen. Since von Wolzogen Kühr (1932) had previously shown that a member of this group, *Pseudomonas fermentans*, gave a positive Voges-Proskauer reaction, the existence of a butylene glycol fermentation in the genus was suspected, although the absence of exact data on the products of fermentation left this point unsettled.

During the course of an intensive investigation on the bacteriological production of 2:3-butylene glycol (Adams & Stanier, 1944), it seemed desirable to make a somewhat closer study of this little-known *Aeromonas* fermentation. The recent discovery of polar flagellation in *Proteus hydrophilus* (Kulp & Borden, 1942; Guthrie & Hitchner, 1943), taken in conjunction with its fermentative abilities, mark this organism as an *Aeromonas* species, and since cultures were readily available it was chosen as a representative of the group.

## MATERIALS AND METHODS

Through the kindness of Dr W. L. Kulp and Dr E. R. Hitchner we obtained a number of strains of *Aeromonas hydrophila*. Preliminary analyses having shown that all these organisms were capable of producing substantial amounts of ethanol and 2:3-butylene glycol from sugars, a strain with particularly vigorous fermentative properties was selected for more detailed quantitative investigations.

The basal medium employed consisted of yeast extract 1·25 g., $CaCO_3$ 1·0 g., $MgSO_4$ 0·025 g., 1·0 M-phosphate buffer (pH 7·4) 0·65 ml., tap water 250 ml. The carbohydrates used were sterilized separately in concentrated aqueous solution and added to the basal medium just before inoculation in amounts giving a final concentration of 1–2%. Fermentations were carried out at 30° and allowed to run for 3–4 days. During this period a slow stream of oxygen-free nitrogen was passed through the flasks. At the end of the fermentation, an excess of HCl was added to dissolve the residual $CaCO_3$, and aeration with nitrogen was continued for several hours to sweep out all the $CO_2$.

Carbon dioxide was determined gravimetrically. Hydrogen was continuously oxidized by passage over heated copper oxide and determined as water. After completion of the fermentation, the fermented solution was neutralized and made up to 500 ml. A measured fraction was made alkaline and extracted continuously with ether in order to remove butylene glycol and acetoin. The ether extract was evaporated, and the residue taken up in water. Butylene glycol was determined by an unpublished method developed in the Department of Biochemistry at the University of Wisconsin. The butylene glycol is oxidized with periodic acid, and the excess periodate determined iodometrically in neutral solution. Acetoin was estimated by the method of Langlykke & Peterson (1937). After extraction of the alkaline solution with ether, it was made acid and re-extracted with ether to remove the organic acids, which were taken up in water again after removal of the ether. The acid extract was steam distilled and the volatile portion estimated as acetic acid by titration. The non-volatile fraction contained lactic and succinic acids. The former was determined by the method of Boyland (1928), the latter by the formation of the silver salt. Pyruvic acid, when present, was withheld from going into the ether acid extract by saturating the solution with sodium bisulphite. Ethanol was determined by dichromate oxidation, following fractional distillation of a measured fraction of the fermentation solution.

Pyruvic acid was determined manometrically by ceric sulphate oxidation (Krebs & Johnson, 1937). Xylose estimations were carried out by the copper reduction method of Schoorl (1929). Glucose was determined by the Hanes (1929) modification of the Hagedorn-Jensen procedure.

## RESULTS

Carbon balances were obtained for the fermentations of glucose, xylose and pyruvic acid. An attempt was also made to study the fermentation of mannitol but, largely owing to the slow and incomplete attack on this compound, a satisfactory balance was not obtained.

*Fermentation of glucose.* The products of glucose fermentation by *A. hydrophila* are shown in Table 1. The main organic ones are always butylene glycol, ethanol and lactic acid; in addition, smaller amounts of acetoin, acetic acid and succinic acid are found. Fluctuations in the quantitative relationships between the various products may occur from one experiment to the next; thus, $CO_2$ production can vary from 140 to 170 mol. per 100 mol. of glucose fermented, with concomitant differences in the relative amounts of $C_2$ and $C_3$ compounds. The ratio of butylene glycol to ethanol can also fluctuate over quite a wide range, although it is usually about 1 : 1 on a molecular basis.

In order to establish beyond doubt the nature of the glycol produced in this fermentation, a small sample was isolated and characterized. About 15 l. of a fermented yeast extract-glucose medium were

Table 1. *Fermentation of glucose by* Aeromonas hydrophila

Glucose fermented: 5·490 g.

| Products | Weight (g.) | Number of mol./100 mol. of glucose fermented: Product | Carbon | Calculated $CO_2$ | Calculated $H_2$ | Oxidation values: + | Oxidation values: − |
|---|---|---|---|---|---|---|---|
| 2:3-Butylene glycol | 1·502 | 54·7 | 218·8 | 109·4 | 54·7 | — | 164·1 |
| Acetoin | 0·047 | 1·7 | 6·8 | 3·4 | 3·4 | — | 3·4 |
| Ethanol | 0·730 | 52·0 | 104·0 | 52·0 | — | — | 104·0 |
| Acetic acid | 0·085 | 4·6 | 9·2 | 4·6 | 9·2 | — | — |
| Lactic acid | 0·639 | 23·3 | 69·9 | — | — | — | — |
| Succinic acid | 0·130 | 3·6 | 14·4 | −3·6 | −3·6 | 3·6 | — |
| $CO_2$ | 2·230 | 166·2 | 166·2 | — | — | 332·4 | — |
| $H_2$ | 0·035 | 57·5 | — | — | — | — | 57·7 |
| Total | — | — | 589·3 | 165·8 | 63·7 | 336·0 | 329·2 |

Carbon recovery ... ... ... 98·2%
$CO_2$ calculated/$CO_2$ observed ... 0·997

$H_2$ calculated/$H_2$ observed ... 1·108
O/R index ... ... ... 1·021

evaporated to a volume of 4 l. in an open steam kettle, saturated with anhydrous $K_2CO_3$, and extracted with twice the volume of butanol. Most of the butanol was removed by distillation through a short Vigreux column, the residual extract was distilled under reduced pressure in a Stedman column and the pure glycol fraction recovered. Oxidation of a small sample of this glycol with bromine and $FeCl_3$ gave a good yield of diacetyl, which was characterized by the melting-point of its bis-phenyl hydrazone. The production of diacetyl on oxidation proved conclusively that the glycol was 2:3-butylene glycol. Judging from its optical rotation (−0·97°), it consists of a mixture of the *l*- and meso-forms with a preponderance of the latter. Thus it differs both from the 2:3-butylene glycol produced in the *Aerobacter* fermentation, which is a *d*-meso mixture, and from that produced in the *Aerobacillus* fermentation, which is the pure *l*-isomer.

*Fermentation of xylose.* Table 2 presents the carbon balance for a xylose fermentation by *A. hydrophila*. In this particular experiment the butylene glycol-ethanol ratio was somewhat less than 1:1, but this is not characteristic; ratios of 1:1 or over have been obtained in other similar experiments. Thus there are no significant differences, either qualitative or quantitative, between the end-products of pentose and hexose fermentation by *A. hydrophila*. This indicates very strongly that there must be a resynthesis from the xylose (or from the $C_2$ fragments of the xylose) of a $C_6$ compound which is subsequently decomposed in a manner similar to that operative in glucose dissimilation. Unless one is willing to assume the production of $CO_2$ from a $C_2$ compound—a reaction unknown in fermentative processes—a simple calculation shows the necessity for this assumption. Were the end-products of pentose fermentation derived directly from the fragments of a $C_3$-$C_2$ split, the $CO_2$ production could not exceed 100 mol./100 mol. of pentose fermented, since no $CO_2$ could be derived from the $C_2$ fraction. Furthermore, the production of $C_3$ compounds will reduce this possible maximum of $CO_2$ production by an amount equivalent to the number of mols of $C_3$ compound formed. In the experiment shown in Table 2, the observed $CO_2$

Table 2. *Fermentation of xylose by* Aeromonas hydrophila

Xylose fermented: 4·010 g.

| Products | Weight (g.) | Number of mol./100 mol. of xylose fermented: Product | Carbon | Calculated $CO_2$ | Calculated $H_2$ | Oxidation values: + | Oxidation values: − |
|---|---|---|---|---|---|---|---|
| 2:3-Butylene glycol | 0·939 | 39·0 | 156·0 | 78·0 | 39·0 | — | 117·0 |
| Acetoin | 0·061 | 2·6 | 10·4 | 5·2 | 5·2 | — | 5·2 |
| Ethanol | 0·600 | 48·9 | 97·8 | 48·9 | — | — | 97·8 |
| Acetic acid | 0·150 | 9·3 | 18·6 | 9·3 | 18·6 | — | — |
| Lactic acid | 0·491 | 20·4 | 61·2 | — | — | — | — |
| Succinic acid | 0·036 | 1·1 | 4·4 | −1·1 | −1·1 | 1·1 | — |
| $CO_2$ | 1·584 | 134·7 | 134·7 | — | — | 269·4 | — |
| $H_2$ | 0·029 | 53·9 | — | — | — | — | 53·9 |
| Total | — | — | 483·1 | 140·3 | 61·7 | 270·5 | 273·9 |

Carbon recovery ... ... ... 96·6%
$CO_2$ calculated/$CO_2$ observed ... 1·041

$H_2$ calculated/$H_2$ observed ... 1·126
O/R index ... ... ... 0·988

Table 3. *Fermentation of pyruvic acid by* Aeromonas hydrophila

Pyruvic acid fermented: 3·530 g.

| | | Number of mol./100 mol. of pyruvic acid fermented | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | Oxidation | |
| | Weight (g.) | Product | Carbon | Calculated $CO_2$ | Calculated $H_2$ | + | − |
| Pyruvic acid fermented | 3·530 | — | — | — | — | — | 100 |
| Products | | | | | | | |
| 2:3-Butylene glycol | 0·125 | 3·5 | 14·0 | 7·0 | −3·5 | — | 10·5 |
| Acetoin | 0·021 | 0·5 | 2·0 | 1·0 | — | — | 1·0 |
| Ethanol | 0·038 | 2·1 | 4·2 | 2·1 | −2·1 | — | 4·2 |
| Acetic acid | 1·800 | 74·8 | 149·6 | 74·8 | 74·8 | — | — |
| Lactic acid | 0·776 | 21·5 | 64·5 | — | −21·5 | — | — |
| $CO_2$ | 1·479 | 83·8 | 83·8 | — | — | 167·6 | — |
| $H_2$ | 0·035 | 43·6 | — | — | — | — | 43·6 |
| Total | — | — | 318·1 | 84·9 | 47·7 | 167·6 | 159·3 |

Carbon recovery ... ... ... 106·3%
$CO_2$ calculated/$CO_2$ observed ... 1·013
$H_2$ calculated/$H_2$ observed ... 1·102
O/R index ... ... ... 1·052

production amounted to 135 mol./100 mol. of xylose fermented: i.e. 35 mol. more than the possible maximum from a $C_3$-$C_2$ split and 58 mol. more than the amount to be expected after taking into account the lactic and succinic acid formed. It would be interesting to determine the effect of *A. hydrophila* on glycol aldehyde, the $C_2$ compound which one might expect to result from the primary fission of the pentose molecule.

*Fermentation of pyruvic acid. Aeromonas hydrophila* causes a vigorous fermentation of pyruvic acid. The balance sheet for this fermentation is shown in Table 3. Surprisingly enough, the production of butylene glycol, acetoin and ethanol is largely suppressed, and the chief organic end-products are acetic and lactic acids. Approximately three molecules of pyruvic acid are oxidized to acetic acid for every one reduced to lactic acid, and consequently a considerable volume of hydrogen is produced. The three main reactions appear to be:

$$3CH_3.CO.COOH + 3H_2O \rightarrow 3CH_3.COOH + 3CO_2 + 6H,$$
$$CH_3.CO.COOH + 2H \rightarrow CH_3.CHOH.COOH,$$
$$4H \rightarrow 2H_2.$$

Thus the dismutation of pyruvic acid by *A. hydrophila* belongs to the type demonstrated for heterofermentative lactic acid bacteria (Nelson & Werkman, 1936) and for staphylococci (Krebs, 1937), with the difference that in the latter fermentations there is an equimolecular production of the two acids and consequently no hydrogen formation.

The dissimilation of pyruvic acid by *A. hydrophila* differs notably from that which occurs in the other two bacterial groups where one finds a butylene glycol fermentation. When growing anaerobically on pyruvic acid, *Aerobacillus polymyxa* produces principally acetoin and acetic acid (Adams & Stanier, 1944), while under similar conditions *Aerobacter aerogenes* gives rise to butylene glycol and acetic acid (Mickelson, Reynolds & Werkman, 1936).

## DISCUSSION

These investigations have definitely established the existence of a butylene glycol fermentation in a bacterial group where it was previously not known. Thus there are at present three morphologically widely separated bacterial families—the Enterobacteriaceae (*Aerobacter* and *Serratia*), the Bacillaceae (*Aerobacillus*) and the Pseudomonadaceae (*Aeromonas*) in which a butylene glycol fermentation occurs. In each family there are also organisms which carry out entirely different carbohydrate fermentations; in the Pseudomonadaceae, the alcoholic fermentation of *Zymomonas lindneri*: in the Enterobacteriaceae, the mixed acid fermentation of *Escherichia coli* and other species: and in the Bacillaceae a wide variety of dissimilatory processes (acetone-butanol, acetone-ethanol and other fermentations) performed by various organisms.

This absence of correlation between morphological and biochemical characters is of considerable interest from the standpoint of comparative biochemistry. It suggests very strongly that the present fermentative mechanisms for the dissimilation of carbohydrates have developed independently along similar lines in different branches of the bacteria. This hypothesis is supported by the slight but significant differences which characterize a particular fermentation as carried out by organisms belonging to different morphological groups. A good example is the differences in optical rotation of the butylene glycols produced by *Aerobacter*, *Aeromonas* and *Aerobacillus*.

## SUMMARY

1. *Aeromonas hydrophila*, a typical representative of the genus *Aeromonas* Kluyver and van Niel, causes a butylene glycol fermentation of carbohydrates. The end-products from glucose are 2:3-

butylene glycol, ethanol, acetic and lactic acids, $CO_2$ and $H_2$, with traces of acetoin and succinic acid. The 2:3-butylene glycol is a mixture of the *l*- and meso-forms.

2. The products of xylose fermentation are very similar, both quantitatively and qualitatively, to those of glucose fermentation. This is regarded as evidence against a simple $C_3$-$C_2$ split of the pentose molecule.

3. The main products of the fermentation of pyruvic acid are lactic and acetic acids, $CO_2$ and $H_2$. Approximately three molecules of pyruvic acid are oxidized to acetic acid for every one reduced to lactic acid.

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# Experiments on the Methylation and Acetylation of Wool, Silk Fibroin, Collagen and Gelatin


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In a previous paper (Blackburn, Carter & Phillips, 1941) it was shown that wool and silk fibroin could be *O*-methylated either by contact with methyl bromide or iodide at room temperatures, or with methyl sulphate in buffers within the pH range 2·5–8·5. With both proteins the number of $CH_3$ groups introduced by repeated treatment with methyl sulphate was greater than could be accommodated on the free carboxyl groups of glutamic and aspartic acids, and it was suggested that certain 'activated' peptide linkages methylated as well as the free carboxyl groups of the salt-linkages. $CH_3I$ and $CH_3Br$ introduced fewer $CH_3$ groups into wool and silk fibroin than did methyl sulphate.

The present paper describes the extension of this work to the methylation of collagen and gelatin by similar methods, and the methylation with methyl sulphate and $CH_3Br$ of wool which has been chemically modified by alkali, acid, nitrous acid and formaldehyde. In addition, the simultaneous acetylation and methylation of wool, silk fibroin and collagen by the combined action of methanol and acetic anhydride has been investigated.

## METHODS

### *Methylation of collagen and gelatin with methyl sulphate and methyl halides*

The collagen used in these investigations was a limed hide which had been delimed with acetic acid and then dehydrated in acetone. The gelatin was Coignet's Gold Label. The methylation procedure was similar to that described for wool and silk (Blackburn *et al.* 1941), with certain modifications to suit the properties of collagen and gelatin. Both proteins were cut into small pieces and acetate buffer was used for the methylations with methyl sulphate. At the end of each period of methylation about 1 g. of collagen was removed, washed in three changes of water and then shaken mechanically with water (25 ml.) for 1 hr. It was then dehydrated with acetone, air-dried and conditioned.* The moisture content of the collagen, the physical properties of which appeared to be unaltered, was determined by Barritt & King's method (1926). During methylation, the gelatin absorbed water and swelled greatly. The resulting gel and solution was dialyzed in cellophan against water for 3 days, the water being changed frequently. The resulting gelatinous mass was dried *in vacuo* below 60°.

Before methylation with either $CH_3Br$ or $CH_3I$, the collagen was allowed to reach equilibrium with a borate buffer of pH 8, and then dehydrated with acetone and conditioned. The gelatin was treated dry as bought. After methylation, both proteins were rinsed in several changes of benzene and then conditioned. When proteins are methylated by either $CH_3Br$ or $CH_3I$, one halogen anion is liberated for each $CH_3$ group introduced. To provide an additional measure of their degree of methylation, the halogen content of some of the methylated collagens and gelatins was therefore determined by the open Carius method (Peters & Van Slyke, 1932). The methylated gelatin

* All proteins described in this paper as conditioned had been exposed to a controlled atmosphere of 21° and 70% R.H. until they attained a constant weight. Although all the analyses were made on the conditioned proteins, the results are recorded as percentages of the anhydrous material.

was also analyzed for N by the Kjeldahl method. The $CH_3$ contents, determined by Baernstein's method (1932, 1936), were then calculated on the dry weight of gelatin, assuming the N content of gelatin to be 17·9%.

### *Methylation of chemically modified wools with methyl sulphate and methyl bromide*

Unless otherwise stated, the wool used was similar to that described previously (Elsworth & Phillips, 1938). The chemically modified wools in which we are at present interested are those in which the chemical modification is restricted to certain groups in the keratin molecule. They were prepared as described below by methods unlikely to cause widespread decomposition of the wool.

*Alkali-treated wool.* Wool (100 g.) was boiled for 30 min. in 10 l. of 2% borax. It was then rinsed repeatedly in water, dried and conditioned. By this treatment the total-S (3·35%) and disulphide-S (2·88%) were reduced to 2·75 and 1·26% respectively, cystine being converted into lanthionine (Horn, Jones & Ringel, 1941, 1942 *a*, *b*; Mizell & Harris, 1943). The only other change likely to have been produced is the degradation of the serine and threonine residues, since those of silk have been shown by Nicolet, Shinn & Saidel (1942) to be susceptible to decomposition by alkalis. Analysis by the methods of Nicolet & Shinn (1941), Shinn & Nicolet (1941) showed, however, that a merino wool which originally contained 9·7% of serine still contained 9·3% after being boiled for 2 hr. in borate buffer at pH 9·6.

*Acid-treated wool.* The wool (100 g.) was boiled for ½ hr. with 1500 ml. of 0·5 N-HCl. The acid was replaced by 1500 ml. of fresh acid with which the wool was boiled for a further 15 min., then washed in several changes of distilled water, dried and conditioned. The amide-N of the wool had been reduced from 1·26 to 0·78%. These determinations were made by the method of Bailey (1937) and Lugg (1938) except that, in order to dissolve the wool completely, the period of hydrolysis was increased from 3 hr. to 4 or 5 hr. The acid-treated wool was brought to pH 5·4 in acetate buffer to remove HCl before methylation.

*Deaminated wool.* This was prepared by Bowes & Pleass's method (1939*a*), which deaminates only the terminal amino-groups of lysine.

*Formaldehyde-treated wool.* The action of formaldehyde on wool can be restricted to the terminal amino-groups of lysine and arginine or can include reaction with the disulphide-S of cystine (Middlebrook & Phillips, 1942). When the reaction was restricted to the amino-groups by treating the wool at ordinary temperature, it contained 1·14% formaldehyde, as determined by Bowes & Pleass's method (1939*b*), and acquired 1·7% $CH_3$ on methylation with methyl sulphate in phosphate buffer. Analysis then showed it was free from formaldehyde. A second sample of wool was treated with 50 times its weight of a 1% solution of formaldehyde at pH 6·9 and 70° for 18 hr. By this treatment its disulphide-S content was reduced from 2·80 to 2·49%.

The chemically treated wools, with the exception of the acid-treated wool, which was methylated in acetate buffer, were methylated with methyl sulphate in phosphate buffer, and also by exposure to $CH_3Br$ after they had been buffered to pH 8·0 in borate buffer, 0·4 M with respect to $H_3BO_3$, and then air-dried (Blackburn *et al.* 1941).

### *Acetylation of wool with acetic anhydride*

Air-dry wool was boiled under reflux with sufficient acetic anhydride to cover it completely. The reaction was allowed to proceed for either 30 min. or for 4 hr., the acetic anhydride was poured off the wool, which was soaked and rinsed in water until free from acetic anhydride, and then air-dried and conditioned. Wool acetylated for 30 min. in this manner was light yellow in colour. It gave a negative Millon reaction. When the wool was acetylated for 4 hr., it became deep brown in colour, but retained a considerable amount of its strength. The total acetyl content of wool acetylated for ½ hr. was 11·7%, and 13·8% after acetylation for 4 hr. Two further samples of wool acetylated for ½ and 4 hr. had total acetyl 12·6% (*O*-acetyl, 2·2%) and total acetyl 14·8% (*O*-acetyl 2·8%) respectively. A small amount of wool dissolved, giving the acetic anhydride a pale yellow colour.

#### *Determination of acetyl groups*

(*a*) *Total acetyl.* The wool (0·30 g.) was boiled under reflux for 4 hr. with 5 N-$H_2SO_4$ (10 ml.). The hydrolysate was distilled *in vacuo*, water being added from time to time, until 200 ml. of distillate had collected. This was titrated, whilst protected from atmospheric $CO_2$, with 0·02 N-NaOH. A sample of untreated wool was analyzed in a similar manner and the blank titration obtained was subtracted from the titrations of the distillates from the acetylated wools.

(*b*) *O-acetyl.* This approximate determination was based on the observations of Wolfrom, Konigsberg & Soltzberg (1936) and Synge (1939) that *O*-acetyl groups, in contrast to *N*-acetyl groups, are readily hydrolyzed by cold dilute NaOH. The acetylated and untreated wools (0·30 g. of each) were immersed in 20 ml. of 0·02 N-NaOH at room temperature. After 24 hr., 10 ml. of the alkali were removed and titrated with HCl against phenol red. The difference in the titrations of the alkali from the acetylated and untreated wools was taken as a measure of the *O*-acetyl groups in the acetylated wools.

### *Simultaneous acetylation and methylation of wool, silk fibroin and collagen with methanol and acetic anhydride*

Ashley & Harington (1929) observed that thyroxine in ethanol at room temperature was converted into the ethyl ester of diacetyl thyroxine by the addition of acetic anhydride: phenylalanine similarly gave the ethyl ester of *N*-acetylphenylalanine. We have found that wool, silk fibroin and collagen can be simultaneously acetylated and methylated in a similar manner. The wool and silk fibroin (3 g.) were dried in a current of air at 115° for 10 min.; the collagen was dehydrated with methanol. They were then placed in methanol (100 ml.) and acetic anhydride (10 ml.) added. After 20 hr., the proteins were rinsed with water, allowed to stand for several hours in water which was changed frequently, dried at room temperature and finally conditioned.

### *Methylation of wool and silk fibroin with methyl* p*-toluenesulphonate*

The wool or silk fibroin (3 g.) was dried in a stream of air at 115° and then heated under reflux with a solution of methyl *p*-toluenesulphonate (10 g.) in methanol (100 ml.)

Table 1. *Analyses of the products of methylation of collagen and gelatin with methyl sulphate and methyl halides*

| Methylation with methyl sulphate | | Methylation with $CH_3I$ | | | Methylation with $CH_3Br$ | | |
|---|---|---|---|---|---|---|---|
| No. of methylations | $CH_3$ (%) | Total time of exposure (days) | $CH_3$ (%) | I (%) | Total time of exposure (days) | $CH_3$ (%) | Br (%) |
| | | | (*a*) Collagen | | | | |
| 2 | 0·56 | 5 | 0·34 | — | 10 | 0·85 | 5·42 |
| 6 | 1·17 | 11 | 0·42 | — | 76* | 0·96 | 6·74 |
| 8 | 1·22 | 72 | 0·54 | 4·92 | 101 | 1·20 | — |
| 12 | 1·11 | 42* | 0·67 | 5·76 | — | — | — |
| 14 | 1·27 | 69* | 0·86 | — | — | — | — |
| 16 | 1·42 | 103 | 0·91 | — | — | — | — |
| | | | (*b*) Gelatin | | | | |
| 10 | 1·74 | 15 | 0·44 | 5·66 | 15 | 1·09 | 8·15 |

* Collagen rebuffered and methyl halide renewed after determination of $CH_3$ had been made.

for 6 hr. The methylated proteins were squeezed, air-dried, extracted with methanol, dried, rinsed for 2·5 hr. in several changes of water, air-dried, and finally conditioned.

*Methylation of wool and silk fibroin with methyl p-toluenesulphonate in the presence of alkalis*

Wool or silk fibroin (6 g.) was dried in a stream of hot air at 115° and then heated under reflux for 6 hr. with methanol (200 ml.) to which methyl *p*-toluenesulphonate (20 g.) and anhydrous potassium carbonate (10 g.) had been added. The methylated proteins were squeezed, rinsed in water, acidified with acetic acid, air-dried, extracted with benzene, air-dried, rinsed in several changes of water, air-dried and finally conditioned.

## RESULTS

*Methylation of collagen, gelatin and chemically modified wools with methyl sulphate and methyl halides*

As can be seen from Table 1, after 16 methylations with methyl sulphate, collagen contained 1·42 % $CH_3$, whilst gelatin, after 10 methylations, contained 1·74 % $CH_3$.

Gelatin contains 5·8 % glutamic acid, 3·4 % aspartic acid, 0·4 % $NH_3$-N and 0·97 % methionine (Dakin, 1920; Baernstein, 1932). Assuming that the $NH_3$-N isolated by Dakin (1920) is all present as amide-N, the methylation of the free carboxyl groups of glutamic and aspartic acids and the S-methyl group of methionine should together account for

$$(0{\cdot}59 + 0{\cdot}38 - 0{\cdot}35 + 0{\cdot}10) = 0{\cdot}72\ \%\ CH_3,$$

or 0·82 % $CH_3$ if the S-methyl group is converted into a sulphonium group. Since collagen takes up 1·42 % $CH_3$ and gelatin 1·74 % $CH_3$, when they are repeatedly methylated with methyl sulphate, some of the $CH_3$ groups become attached to centres other than the free carboxyl groups.

Table 2. *Analyses of the products of methylation of chemically modified wools with methyl sulphate and methyl bromide*

| Methylation with methyl sulphate (in phosphate buffer) | | Methylation with $CH_3Br$ | | |
|---|---|---|---|---|
| No. of methylations | $CH_3$ (%) | Total time of exposure (days) | $CH_3$ (%) | Br (%) |
| | | (*a*) Alkali-treated wool | | |
| 2 | 0·82 | 28* | 1·19 | 9·77 |
| 5 | 1·07 | 73† | 0·94 | — |
| 9 | 1·55 | 114 | 1·51 | — |
| 14 | 1·69 | — | — | — |
| 16 | 1·45 | — | — | — |
| 18 | 1·68 | — | — | — |
| | | (*b*) Acid-treated wool | | |
| 1 | 0·39 | 22* | 1·09 | 11·25 |
| 2 | 0·94 | 68† | 1·00 | — |
| 3 | 1·41 | 109 | 1·50 | — |
| 6 | 2·08 | — | — | — |
| 9 | 2·39 | — | — | — |
| 11 | 2·74 | — | — | — |
| 12 | 2·90 | — | — | — |
| | | (*c*) Deaminated wool | | |
| 2 | 0·64 | 20* | 1·16 | 7·18 |
| 5 | 1·25 | 58† | 0·92 | — |
| 8 | 1·73 | 99 | 1·35 | — |
| 11 | 1·66 | — | — | — |
| 14 | 1·61 | — | — | — |
| | | (*d*) Formaldehyde-treated wool | | |
| 2 | 0·59 | 14† | 0·94 | 8·20 |
| 5 | 1·17 | 28 | 1·16 | — |
| 9 | 1·47 | — | — | — |
| 13 | 1·64 | — | — | — |
| 15 | 1·51 | — | — | — |
| 17 | 1·58 | — | — | — |

* $CH_3Br$ renewed after determination of $CH_3$ was made.

† Wool rebuffered and $CH_3Br$ renewed after determination of $CH_3$ had been made.

The analyses of the chemically treated wools given in Table 2 show that mild alkali treatment, deamination and treatment with HCHO at 70° did not alter the degree to which the wool could be methylated with methyl sulphate. On the assumption that some methylation with methyl sulphate occurred at 'activated' peptide linkages, it would appear that these linkages were unaffected by the treatments given. Deamination and treatment with HCHO, under the experimental conditions employed, would only affect the terminal amino groups of the lysine side-chains. These are few in number (corresponding to about 0·25% $CH_3$) and this probably accounted for the small effect of both deamination and treatment with HCHO on the observed degree of methylation with either methyl sulphate or $CH_3Br$.

The acid treatment given, however, enabled the wool to take up 1·0% extra $CH_3$ when methylated with methyl sulphate. This may have been due partly to the esterification of carboxyl groups liberated from amide groups (the decrease in amide-N was equivalent to 0·51% $CH_3$) and partly to the esterification of carboxyl groups liberated by the hydrolysis of the main chains. Experiment showed that, when wool was boiled for 30 min. with 0·5N-HCl, at least 70% of the N passing into solution was $NH_3$-N: the amide-N of the wool showed a corresponding decrease. In contrast to methyl sulphate, $CH_3Br$ did not methylate acid-treated wool to a higher degree than it does untreated wool.

*Methylation of acetylated wool*

When wool which had been acetylated for 30 min. was repeatedly methylated with methyl sulphate in phosphate buffer, the $CH_3$ introduced appeared to reach a limiting value of 0·9% (Table 3). The even lower limit of 0·6% $CH_3$ appeared to be reached when wool acetylated for 4 hr. was methylated, although 1·7% $CH_3$ can be introduced into untreated wool by 18 successive methylations (Blackburn *et al.* 1941). Methylation by the methyl halides was also greatly restricted.

*The conversion of arginine into ornithine during the acetylation of wool*

By Vickery's method (1940), 19·7% of the total N of the hydrolysate of the untreated wool was found to be arginine-N. On the other hand, only 9·5% of the total N of the hydrolysate of a wool which had been acetylated for 30 min. was arginine-N. It was evident therefore that arginine is destroyed when wool is acetylated.

On adding flavianic acid to the hydrolysate of a wool (50 g.) which had been acetylated for 4 hr., the precipitate obtained, which separated slowly during 2 weeks in a refrigerator, was found to be free from arginine diflavianate and to consist entirely of ornithine flavianate. This was recrystallized from hot water, dissolved in dilute HCl and the flavianic acid extracted with *n*-butanol (Pratt, 1926). The aqueous solution containing the base was concentrated under reduced pressure and decolorized with charcoal. The benzoyl derivative, prepared by the addition of benzoyl chloride and NaOH, after recrystallization from aqueous ethanol had m.p. 185–186° either alone or when mixed with *N*:*N'*-dibenzoyl ornithine (Boon & Robson, 1935; Karrer & Ehrenstein, 1926). (Found: N, 8·6. Calc. for $C_{19}H_{20}O_4N_2$: N, 8·25%.) It was optically inactive to light of $\lambda$ 5893 in 0·1N-NaOH (*c*, 0·91; *l*, 50 mm.). Only 0·75 g. of dibenzoyl ornithine was isolated from the 50 g. of acetylated wool, but Vickery (1941) also found the isolation of ornithine from protein hydrolysates presents very special difficulties.

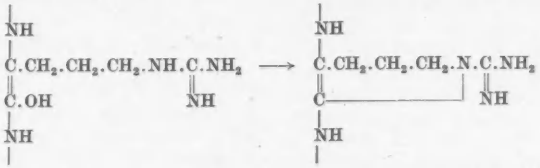
Since arginine is destroyed when wool is acetylated, it is reasonable to suppose that the ornithine isolated is derived from this source. Bergmann & Koster (1926) found that, on being heated with excess acetic anhydride, arginine was converted

Table 3. *Analyses of the products of methylation of acetylated wools with methyl sulphate and methyl halides*

| Methylation with methyl sulphate | | Methylation with $CH_3I$ | | | Methylation with $CH_3Br$ | | |
|---|---|---|---|---|---|---|---|
| No. of methylations | $CH_3$ (%) | Total time of exposure (days) | $CH_3$ (%) | I (%) | Total time of exposure (days) | $CH_3$ (%) | Br (%) |
| | | (*a*) Wool acetylated for ½ hr. | | | | | |
| 2 | 0·35 | 14 | 0·27 | — | 14 | 0·34 | 3·34 |
| 5 | 0·60 | 35 | 0·38 | — | 35 | 0·44 | 4·29 |
| 9 | 0·70 | — | — | — | — | — | — |
| 14 | 0·78 | — | — | — | — | — | — |
| 18 | 0·86 | — | — | — | — | — | — |
| | | (*b*) Wool acetylated for 4 hr. | | | | | |
| 3 | 0·42 | 14 | 0·16 | — | 14 | 0·19 | 2·64 |
| 7 | 0·57 | 35 | 0·27 | — | 35 | 0·35 | 3·76 |
| 14 | 0·64 | — | — | — | — | — | — |
| 16 | 0·57 | — | — | — | — | — | — |

into triacetyl anhydro-arginine, which with cold water gave immediately *dl*-acetamido-2-piperidone and diacetyl urea. Acid hydrolysis of the piperidone derivative gave *dl*-ornithine.

Dirr & Felix (1932) considered that the conversion of combined arginine into ornithine only took place during acetylation when the carboxyl group of the arginine was free (compare Bergmann & Koster, 1927; Felix & Dirr, 1928). If the assumption of Dirr & Felix (1932) is correct, then our experiments suggest that some of the carboxyl groups of the arginine in wool are also free. The available evidence is against this conclusion (Dauphinee & Hunter, 1930). In addition, the assumption of Dirr & Felix (1932) would seem to be unnecessary to account either for their own results with clupein or our results with wool, as ring closure not involving a free carboxyl group might occur if the peptide link adjacent to the arginine residues assumed the enolic form. Such enolization might cause loss of asymmetry of the α-carbon atom of the arginine and, as in our experiments, an optically inactive ornithine would be liberated by acid hydrolysis.



If ring closure occurs in this manner, the terminal amidine group would probably acetylate and on hydrolysis would be eliminated either as acetyl urea or its decomposition products.

*The simultaneous acetylation and methylation of wool, silk fibroin and collagen with methanol and acetic anhydride*

Table 4 shows that, when these proteins were treated with methanol-acetic anhydride, both $CH_3$ and acetyl groups were introduced.

Table 4. *Analyses of the products of the simultaneous acetylation and methylation of wool, silk fibroin and collagen with methanol and acetic anhydride*

| Protein | $CH_3$ (less methionine $CH_3$) (%) | Acetyl (%) Total | Acetyl (%) *O*-acetyl | Molar ratio Total acetyl/$CH_3$ | Molar ratio *O*-acetyl/$CH_3$ |
|---|---|---|---|---|---|
| Wool | 0·53 | 2·5 | 1·9 | 1·6 | 1·2 |
| | 0·73 | — | — | — | — |
| | 0·71 | — | — | — | — |
| Silk fibroin | 0·26 | 1·5 | 1·3 | 2·0 | 1·7 |
| Collagen | 0·64 | — | — | — | — |

The $CH_3$ content (0·79 %) of the wool methylated with methanol-acetic anhydride was unchanged by immersing the wool overnight in phosphate buffer of pH 6 (50 ml./g.) and decreased only to 0·67 % when exposed in a buffer solution to a current of steam. The $CH_3$ groups are therefore chemically combined with the wool. When a treated wool (0·59 % $CH_3$) was again treated with acetic anhydride in methanol, the $CH_3$ content rose to 0·75 %. Both *O*- and *N*-acetyl groups are introduced into proteins by methanol-acetic anhydride (Table 4). The acetylated and methylated proteins were turned yellow by nitrous acid at the same rate as were the untreated proteins, suggesting that the tyrosine hydroxyl groups had not been acetylated (Rutherford, Patterson & Harris, 1940). Amino-N determinations on the treated wool (Rutherford, Harris & Smith, 1937) showed that $N_2$ was evolved only very slowly by the action of nitrous acid, indicating that the terminal amino groups of lysine had been acetylated.

Table 5. *Analyses of the products of the further methylation with methyl sulphate and methyl halides of wool which had been acetylated and methylated with methanol and acetic anhydride*

| Methylation with methyl sulphate (phosphate buffer) No. of methylations | Total $CH_3$ (%) | Methylation with $CH_3I$ Time of exposure (days) | Total $CH_3$ (%) | I (%) | Methylation with $CH_3Br$ Time of exposure (days) | Total $CH_3$ (%) | Br (%) |
|---|---|---|---|---|---|---|---|
| 0 | 0·77 | 0 | 0·77 | — | 0 | 0·77 | — |
| 2 | 1·31 | 11 | 1·04 | 4·53 | 11 | 1·01 | 4·72 |
| 4 | 1·35 | 0 | 0·74 | — | 0 | 0·74 | — |
| 6 | 1·48 | 32 | 1·02 | 5·02 | 32 | 1·31 | 5·11 |
| 8 | 1·50 | — | — | — | — | — | — |
| 10 | 1·43 | — | — | — | — | — | — |
| 12 | 1·53 | — | — | — | — | — | — |
| 14 | 1·48 | — | — | — | — | — | — |
| 16 | 1·66 | — | — | — | — | — | — |

It can be seen from Table 5 that when wool which has been treated with methanol-acetic anhydride is methylated with methyl sulphate it takes up further $CH_3$ groups slowly until the total $CH_3$ reaches the value normally attained on methylating wool with this reagent.

### *The methylation of wool and silk fibroin with methyl* p-*toluenesulphonate*

Repeated methylation of wool with methyl *p*-toluenesulphonate in methanol caused the $CH_3$ content to increase gradually to 1·91 %, whilst when silk fibroin was methylated in a similar manner, 0·40 % $CH_3$ was introduced (Table 6). A wool which had been methylated three times with methyl *p*-toluenesulphonate ($CH_3$, 1·91 %) was remethylated with methyl sulphate (Table 7). The $CH_3$ content of the wool reached 3·22 %, which is greater by 1·2–1·5 % than the $CH_3$ content of a wool fully methylated with methyl sulphate.

The nitrous acid test of Rutherford *et al.* (1940) showed the tyrosine hydroxyl groups in the wool had not been methylated, but it was found that after methylation with the sulphonic ester, the wool ($CH_3$, 1·66 %) contained only 0·57 % amide-N as compared with 1·26 % for the untreated wool. Hence some of the observed additional methylation was due to methanolysis of glutamine and asparagine side-chains. The fall in amide-N (0·69 %) is equivalent to 0·74 % $CH_3$. Subtraction of this value from the total $CH_3$ of this wool gives 0·92 % $CH_3$ as the possible amount of $CH_3$ introduced at the other centres.

Table 6. *Analyses of the products of methylation of wool and silk fibroin with methyl* p-*toluenesulphonate*

| Conditions of methylation | | $CH_3$ (%) Wool | $CH_3$ (%) Silk fibroin |
|---|---|---|---|
| 24 hr. at room temp. | | 0·34 | 0·10 |
| 6 hr. under reflux | (*a*) | 1·11 | 0·40 |
| | (*b*) | 1·39 | — |
| Successive treatments for 6 hr. under reflux | (first) | 1·40 | — |
| | (second) | 1·78 | — |
| | (third) | 1·91 | — |

Table 7. *Analyses of the products of the further methylation with methyl sulphate and phosphate buffer of wool which had been methylated with methyl* p-*toluenesulphonate*

| No. of methylations | $CH_3$ (%) |
|---|---|
| 3 | 2·72 |
| 7 | 3·04 |
| 12 | 2·70 |
| 15 | 3·22 |

Table 8. *Analyses of the products of methylation of wool and silk fibroin with methyl* p-*toluenesulphonate in the presence of alkalis*

| Wool: Conditions of methylation | $CH_3$ (%) | Silk fibroin: Conditions of methylation | $CH_3$ (%) |
|---|---|---|---|
| MeOH/$K_2CO_3$ 6 hr. | 1·39 | MeOH/$K_2CO_3$ 6 hr. | 0·97 |
| „ 6 hr. | 1·04 | Water/NaOH 2 hr. | 0·87 |
| „ 1·5 hr. | 0·99 | „ 4 hr. | 1·00 |

The $CH_3$ contents of wool and silk fibroin after treatment with methyl *p*-toluenesulphonate and alkali are given in Table 8. The wool was markedly degraded by the 6 hr. treatment, but when the length of time of treatment was reduced to 1·5 hr., much less degradation took place, although the amount of $CH_3$ introduced was not much lower. Silk fibroin withstood the treatment without physical degradation and was similarly unaffected when the methanol and $K_2CO_3$ were replaced by 0·5 % aqueous NaOH. The nitrous acid test of Rutherford *et al.* (1940) showed that some methylation of the hydroxyl groups of the tyrosine in both the wool and the silk fibroin had taken place.

The results show that methylation with methyl *p*-toluenesulphonate introduces about the same amount of $CH_3$ as is introduced by methyl sulphate, but the reaction is less selective, and even in the absence of alkali protein hydrolysis is brought about by the *p*-toluenesulphonic acid which is liberated.

## DISCUSSION

The $CH_3$ introduced into wool by treatment with methanol-acetic anhydride is limited in amount (about 0·7 %) and is removed slowly, as methanol, by regulated alkaline hydrolysis. Since amino-acids are esterified by this mixture (Ashley & Harrington, 1929), it is probable that it also esterifies the carboxyl groups of the salt-linkages of wool, silk fibroin, and collagen.

By further methylation with methyl halides, the $CH_3$ content of wool, which has been methylated with methanol-acetic anhydride, can be increased to about 1·0 %, but when this value has been reached the methylation either stops or becomes very slow. The $CH_3$ content thus reaches the same value as that usually attained when untreated wool is methylated with methyl halides. It is probable therefore that during the initial relatively rapid methylation of wool with methyl halides the free carboxyl groups are esterified. In agreement with this conclusion, the $CH_3$ introduced by methyl halides is removed only slowly by hydrolysis.

Further evidence that both methanol-acetic anhydride and the methyl halides esterify the free carboxyl groups is provided by comparison (Table 9)

of the $CH_3$ introduced by these reagents with the $CH_3$ equivalent to the free carboxyl groups found by titration and as calculated from the aspartic and glutamic acid contents of wool, collagen and silk fibroin.

Table 9. *The* $CH_3$ *of proteins equivalent to free carboxyl groups as calculated (I) from free carboxyl groups determined from titration curves, (II) from free carboxyl groups of estimated aspartic and glutamic acids and amide-N contents, and as found by (III) methylation with methanol-acetic anhydride and (IV) methylation with methyl halides*

| Protein | I | II | III | IV |
|---|---|---|---|---|
| Wool | 1·17 (1) | 0·96 (4) | 0·75 | 1·0 (7) |
| Collagen | 0·53 (2) | 0·62 (5) | 0·64 | 0·9 |
| Silk | 0·20–0·26 (3) | 0·10 (6) | 0·26 | 0·37 (7) |

(1) Harris & Rutherford (1939); Steinhardt & Harris (1940). (2) Theis & Jacoby (1942). (3) Gleysteen & Harris (1941). (4) Speakman & Townend (1937). (5) Dakin (1920). (6) From diamino-acid content, Vickery & Block (1931). (7) Blackburn *et al.* (1941).

Methanol-acetic anhydride introduces rather less than the expected amount of $CH_3$ into wool, whilst the methyl halides introduce rather more than the expected amounts into all three proteins, but there is no wide disagreement.

When wool is methylated with methyl sulphate more $CH_3$ is introduced than by methylation with either methanol-acetic anhydride or methyl halides. Methylation with methyl sulphate appears to occur in two stages, the first being a relatively rapid initial methylation, after which the $CH_3$ content increases slowly to a maximum of about 1·7%. The $CH_3$ introduced in the earlier stage of the methylation is removed only very slowly by exposing the wool in pH 7 buffer to a current of steam, but by this means the $CH_3$ content of a wool containing 1·63% $CH_3$ can be reduced to 0·72% in 60 min., although a further 180 min. treatment is required to reduce it to 0·55% (Blackburn *et al.* 1941). It thus appears probable that methyl sulphate methylates two centres: the free carboxyl groups which give ester groups that hydrolyze slowly and a second centre which may be an activated peptide linkage that methylates slowly, giving methoxyl groups that are readily removed by hydrolysis.

When this second centre is methylated, sulphate becomes covalently linked to the protein. For example, after 1·66% $CH_3$ had been introduced into wool by 20 methylations with methyl sulphate in phosphate buffer, the wool contained 3·30% $SO_4$ by Mease's method (1934). When samples (2 g.) of this wool were immersed at room temperature in buffers (100 ml.) of pH 0·5–10·5 for 16 hr. only 0·24% $SO_4$ (calc. on the weight of the wool) was extracted by the buffer of pH 0·5: the remaining buffers contained only traces of sulphate. Barritt, Bowen, Goodall & Whitehead (1938) have shown that 0·5% aqueous solution of pyridine will extract all the sulphate from wool which has been steeped in dilute $H_2SO_4$. Experiment showed, however, that a fully methylated wool ($SO_4$, 4·69%) still contained 4·04% $SO_4$ after extraction with this solvent.

That sulphate is introduced into wool during the slower peptide methylation is in agreement with the fixation of sulphate during the methylation by methyl sulphate of acetylated wools and wools methylated with methanol-acetic anhydride. Acetylated wools react very slowly with methyl halides, but they undergo a restricted and slow methylation when treated with methyl sulphate. For example, wools acetylated for 30 min. and 4 hr. contained after 15 methylations in phosphate buffer $CH_3$ 0·95%, $SO_4$ 2·88%, and $CH_3$ 0·67%, $SO_4$ 2·30% respectively. Further, when a wool which had been simultaneously acetylated and methylated with methanol-acetic anhydride was methylated 15 times with methyl sulphate in phosphate buffer, its $CH_3$ content increased from 0·74 to 1·60% and 2·72% sulphate was introduced. The supposed peptide methylation involves the introduction into wool of about 0·7% $CH_3$ and if one assumes that one sulphate group were introduced for each $CH_3$ group, a fully methylated wool should contain 4·5% $SO_4$. Generally less than this amount was found, but this may be because, during the repeated methylations, the wool was in contact with buffer solutions for long periods.

Blackburn *et al.* (1941) suggested the peptide linkage methylated by methyl sulphate was a resonance hybrid of two electronic structures which approached a zwitterion in constitution. Since this suggestion did not explain why sulphate becomes covalently linked to wool during the methylation, it was later suggested (Blackburn, Middlebrook & Phillips, 1942) that oxazoline ring structures are formed by condensation between the hydroxy side-chains of serine and threonine and the activated peptide linkages. It was further suggested that during methylation with methyl sulphate cleavage of the oxazoline rings so produced took place, the enolized peptide linkage being *O*-methylated and the hydroxy groups of the liberated serine and threonine side-chains sulphated. The experimental evidence given above lends some support to these suggestions.

## SUMMARY

1. When collagen and gelatin are methylated with methyl sulphate, peptide methylation as well as esterification of free carboxyl groups takes place. During peptide methylation, sulphuric acid becomes covalently linked to proteins. When exposed to

methyl iodide or methyl bromide, the free carboxyl groups are esterified, but peptide methylation only occurs slowly, if at all. Neither the peptide methylation nor the esterification of the free carboxyl groups of wool is prevented by treating it before methylation with dilute alkali, nitrous acid or formaldehyde. On the other hand, removal of amide groups by acid hydrolysis leads to increased esterification.

2. When wool is acetylated with acetic anhydride, the subsequent methylation of free carboxyl groups by either methyl sulphate or methyl halides is restricted. Treatment of wool and silk fibroin with acetic anhydride in methanol leads to *N*- and *O*-acetylation, simultaneously with the methylation of free carboxyl groups.

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# Diplococcin, an Anti-bacterial Protein Elaborated by Certain Milk Streptococci

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Whitehead (1933) first showed that milk sometimes contains lactic acid-producing streptococci which inhibit the action of the very similar organisms used industrially as cheese starters. He further proved that the inhibition was due to a protein-like substance produced by the inhibiting organisms. It was not however made clear whether this substance was a constituent of the medium altered by bacterial

action, an excreted metabolic product, or a part of the bacterial cell which had been dissolved by the medium. The problem has recently been investigated on the practical side by Meanwell (1943), and the present author has been able to examine two strains of inhibitory cocci isolated by him. The anti-bacterial effect is in fact due to a protein-like substance of small molecular weight which is normally a constituent of the bacterial cell. It is proposed to call this substance '*Diplococcin*' since the bacteria which produce it exhibit a diplococcal arrangement in stained preparations. The present paper is mainly concerned with the isolation of diplococcin from the bacterial cell.

## EXPERIMENTAL

*Cultures.* Two pure cultures of anti-bacterial streptococci, labelled F 7 and F 8, were received from Mr L. J. Meanwell. These were used for the isolation of diplococcin. The test-organism used was obtained from a cheese starter labelled 303. It was immediately plated out and an isolate made from a single colony. This culture, called 303 S, was used throughout in the routine testing of bacteriostatic material isolated from F 7 and F 8 cultures.

### *Method of assay for anti-bacterial potency*

Stock cultures of 303 S, viz. 2-day growths on heart agar slopes, were kept in the cold room and used after a week. The cultures used in the assay were always 1-day cultures in 2% glucose-broth, derived from a similar culture. A 0·05 or 0·1% solution of the material to be assayed, in 0·4% acetic acid, was sterilized at 100° for 30 min. When a liquid was to be assayed it was first brought to pH 3–4 before sterilization. The reason for this treatment is that diplococcin is not stable at 100° at a pH above 4 (see below).

Two mixtures containing 0·5 and 0·25 ml. of the solution and 4·5 and 4·75 ml. of glucose-broth, respectively, were made aseptically, and further dilutions were made from them, each tube to contain 5 ml., of which at least 4·75 ml. were glucose-broth. Usually a series of eight dilutions was employed to cover the range 10,000–1,000,000. The controls (*A* and *B*) were made correspondingly, the former containing 0·5 ml. and the latter 0·25 ml. of sterile 0·4% acetic acid respectively. All ten tubes were inoculated with one loopful (0·004 ml., containing about 20,000 viable bacteria) of a culture of 303 S. This was made by transferring one loopful of a well-shaken 24-hr. culture in glucose-broth, which had remained 45 min. at room temperature after being shaken, into 4 ml. of sterile glucose-broth and mixing well. After thorough shaking, the inoculated tubes were incubated at 24° and read after 18 and 24 hr. After 18 hr. control *A* should show a faint turbidity and control *B* a good turbidity, whilst after 24 hr. *A* should show a good turbidity and *B* maximum growth with deposit.

*One unit* of anti-bacterial substance was defined as the least amount which, dissolved in 5 ml. of glucose-broth, will prevent visible growth under the above conditions in 18 hr. or, what was almost always the same thing, prevent maximum growth from being attained in 24 hr. In the sequel, the anti-bacterial potency is expressed throughout as units/mg. in the case of solids, and units/ml. in the case of liquids. Thus, the sterilized supernatant obtained from the growth of organisms F 7 or F 8 for one day in glucose-broth usually contained 28 units/ml.

### *Preparation of an active extract from bacterial cells separated from an inactive medium*

Organisms F 7 and F 8 grew poorly in heart broth containing no added carbohydrate, the pH remaining near neutrality. The metabolism solution so obtained was devoid of activity. Whitehead obtained similar results with caseinogen broth, but made no attempt to isolate the anti-bacterial substance from the bacterial cells.

Organism F 7 was grown for 6 days in 3 l. of heart broth; the bacterial cells were spun down and the clear supernatant decanted. The deposit was washed once with distilled water (200 ml.), then dried *in vacuo* at room temperature over $H_2SO_4$ to yield 0·10 g. of dried cells which, after grinding in a glass mortar, were refluxed for several hours with 20 ml. of 0·4% acetic acid (cf. Freeman, 1942). The cooled extract was centrifuged at high speed until almost clear, decanted, heat-sterilized and assayed: *found*, 10 units/ml.

### *Large-scale production of the anti-bacterial substance from the cells*

Since it appeared probable that the anti-bacterial agent could most conveniently be obtained from the cells, it was necessary to find a medium which would allow good growth of organisms F 7 and F 8, but which would not yield any organic precipitate upon acidification, as does undiluted heart broth, to contaminate the bacterial cells. The heavily buffered medium C of Birkinshaw, Charles & Clutterbuck (1931), to which 2·5–5% of heart broth had been added, was found to be admirably suited for this purpose. Sucrose was chosen as carbohydrate source since it could be autoclaved with phosphates at pH 7–8 without appreciable darkening taking place.

The medium had the following composition: $NaNH_4HPO_4$, $4H_2O$, 490 g.; $KH_2PO_4$, 870 g.; $MgSO_4$, $7H_2O$, 50 g.; KCl, 50 g.; $FeSO_4$, $7H_2O$, 1 g.; sucrose, 3 kg.; heart broth, 2·5 or 5 l.; distilled water, about 90 l. The pH was adjusted to 7·8 by the cautious addition of concentrated KOH solution and the volume made up to 100 l. with distilled water. The medium was distributed in 1·5 l. lots in 2 l. conical flasks which were plugged and sterilized by autoclaving. A number of 5 ml. tubes of the above medium was also prepared, inoculated with organism F 7 or F 8, and used after a day's incubation for sowing the flasks, one tube to each flask. The temperature of incubation was 24°.

The following points have been noted: it is not necessary to shake the flasks except immediately after sowing; it is not advantageous to extend the period of incubation beyond 11 days, since further incubation gives no increase in the amount of anti-bacterial substance in the cells, and increases

the risk of mould infection; when the growth is harvested the relatively clear top three-quarters of the medium may be decanted and discarded.

The cells were collected by the use of a De Laval centrifuge. They were washed on the centrifuge by distilled water (500 ml.) and the head of the centrifuge was inverted and allowed to drain before dismantling. All parts which appeared to have bacteria on them were immediately dried *in vacuo* at room temperature over $H_2SO_4$. After 3 days the flakes and scales of dried bacteria were detached without difficulty. It is not necessary to kill the bacteria by heat or otherwise, nor to de-fat them by use of an organic solvent. The dried bacteria were worked up in 5 g. lots after being finely powdered in a mortar. Each lot was first refluxed with 500 ml. of distilled water containing 10 drops of glacial acetic acid for 75 min. (oil-bath at 140°), in order to remove adhering constituents of the medium. After being cooled, the suspension was spun down to yield a virtually inactive supernatant at pH 4. The solid residue was resuspended in 100 ml. of distilled water and re-centrifuged. The deposit was refluxed for 5 hr. with a solution of glacial acetic acid (2·1 ml.) in distilled water (500 ml.). After standing overnight the mixture was spun down in 100 ml. lots at high speed for several hours, and the almost clear supernatant carefully decanted. In general this contained 100 units of activity/ml. A second similar extract of the cells had less than one-fourth of the activity of the first extract.

*Isolation of an active picrate.* The active extract (500 ml.) was treated with Esbach's reagent (150 ml.), with shaking. After some hours the yellow flocculent precipitate was collected on a small Buchner funnel, washed once with a little water, dried *in vacuo* (weight, about 0·3 g.) and assayed: *found*, usually 200 units/mg. Picric acid itself has no activity at a concentration of 1 part in 5000 of glucose-broth. Tannic acid also precipitates the anti-bacterial substance but no satisfactory method for recovery has yet been found.

Table 1 summarizes the large-scale experiments so far carried out.

Table 1. *Production of anti-bacterial substance by organisms* F 7 *and* F 8

| Exp. | Organism and vol. of medium (l.) | Incubation (days) | Yield of dried cells (g.) | Picrate from 5 g. of dried cells (g.) | Activity of picrate (units/mg.) |
|---|---|---|---|---|---|
| A | F7, 94·5 | 10–11 | 23·5 | 0·35–0·39 | 100–200 |
| B | F7, 43·5 | 14–20 | 7·6 | 0·33 | 200 |
| C | F8, 43·5 | 18–24 | 8·8 | 0·28 | 200 |
| D | F7, 45 | 11–13 | 3·7 | 0·16 (from 3·7 g.) | 200 |

All the media contained 5% heart broth except D, which contained 2·5%.

*Decomposition of the picrate to yield an active protein-like substance.* The picrate (0·7 g. very finely powdered) was suspended in absolute ethanol (1050 ml.), and 0·4% acetic acid (70 ml.) added with shaking. The mixture was kept for 3 days in the cold room, with frequent shaking; any visible lumps were broken up from time to time with a glass rod. The deposit was collected by centrifuging and washed on the centrifuge with absolute ethanol until the washings were colourless. The cream-coloured residue (crude diplococcin) weighed 0·4 g. when dry and contained 100 units/mg. This showed that no marked loss of activity had occurred. The residual colour could not wholly be removed by a second ethanol-acetic acid treatment, but there was no appreciable further loss in weight and the activity remained unimpaired. It may be concluded that diplococcin is insoluble in ethanol and that it is not appreciably inactivated by ethanol when it is out of solution at the beginning.

*Properties of crude diplococcin.* Crude diplococcin after two ethanol-acetic acid treatments had: N, 13·9%; S, nil (Weiler, Oxford). It gave almost all the usual protein tests with the exception of Heller's nitric acid and the $HgSO_4$-$H_2SO_4$ precipitation tests and hence is probably less complex than a meta-protein; it is laevo-rotatory, is precipitated by half saturation with ammonium sulphate, gives no Molisch reaction, and diffuses slowly through a collodion membrane. It thus appears to be a protein-like material of relatively small molecular size and may possibly be a proteose. It contains no sulphur or phosphorus and gives reactions for arginine, tyrosine and tryptophan.

*Fractionation of crude diplococcin.* Crude diplococcin can be freed from the remaining undecomposed picrate by treatment with cold distilled water. The almost colourless filtrate so obtained, after removal of coloured insoluble material, yields precipitates with increasing amounts of ethanol, without serious loss of activity if the experiment is conducted at 4°. There is, however, no marked concentration of activity in any one fraction.

Crude diplococcin (100 mg.) was triturated with distilled water (25 ml.) for 30 min. and filtered. The filtrate, cooled to 4°, was treated with ice-cold ethanol (100 ml.) and left in the cold room for 2 hr. The precipitate, collected by centrifuging, weighed 40 mg. and contained 100 units of activity/ml. *Found* (Weiler, Oxford) on this fraction dried at 60° *in vacuo*: C, 50·5; H, 7·3; N, 13·1%; ash, nil. The aqueous-ethanol supernatant from the first precipitate was treated with a further quantity of ice-cold ethanol (550 ml.) and left in the cold room overnight, when a further 0·8 mg. of about the same activity was precipitated. The final clear supernatant was evaporated to 30 ml. *in vacuo* below 40° to yield a further 9 mg. of insoluble material which also contained about 100 units of activity/mg. The liquid residue yielded only 6 mg. of almost inactive material when evaporated to dryness at room temperature over $H_2SO_4$.

Considerable loss of activity occurred when a similar ethanol fractionation was carried out at room temperature in the first stage.

*Extraction of diplococcin from the bacterial cells by cold 0·4% acetic acid*

A possibility exists that extensive degradation of the true anti-bacterial substance of the cells may occur, without loss of activity, during the process of extraction with boiling 0·4% acetic acid. The following experiment definitely shows that this is improbable.

The cells from an experiment similar to D of Table 1, but with an 8-day period of incubation, were collected, washed with distilled water (500 ml.) and resuspended in 0·4% acetic acid (1550 ml.) for 7 days at 24°. After spinning, the clear supernatant (containing 10 units/ml.) was evaporated to one-thirtieth of its volume *in vacuo* below 55°. The residual liquid, which was at pH 4, contained 100 units/ml., did not give the Heller nitric acid reaction for proteins, and appeared in all essential respects to be identical with the previously described extract obtained at 100°. Similar experiments showed that the washed cells would part with a detectable amount of their anti-bacterial substance to a phosphate buffer at pH 4 but not to distilled water at 24°.

*The anti-bacterial substance in metabolism solutions of organisms* F 7 *and* F 8

The above results suggested that a part of the anti-bacterial activity in metabolism solutions had been derived from the cells merely by a process of chemical extraction by a dilute solution of lactic acid buffered at pH 4–5; in other words, that the active substance in the metabolism solution is the same as that extracted from the cells by dilute acetic acid. It is significant that anti-bacterial activity is strongly in evidence only in acidic metabolism solutions.

A portion of the clear supernatant from experiment D of Table 1 (500 ml. containing 40 units/ml.) was treated with Esbach's reagent (140 ml.) and after 24 hr. the liquid was agitated slightly to separate the flocculent picrate from some massive yellow crystals, decanted and centrifuged. The deposit was washed once with water (25 ml.), dried (weight, 10 mg.) and assayed: *found*, 300 units/mg. The picrate had all the properties of diplococcin picrate. The unsown medium, which contained 2·5% of heart broth, gave no flocculent picrate under similar conditions.

*Heat inactivation of diplococcin*

A dilute solution of diplococcin in 0·4% acetic acid loses no activity in 2 hr. at 100°, and very little under the same conditions at pH 4, but is rapidly inactivated by heat on the alkaline side. It is also slowly and irreversibly inactivated at pH 6–7, i.e. when its solution in distilled water or saline is kept in a boiling water-bath.

Crude diplococcin (4·5 mg.) was dissolved in distilled water (9 ml.) by heating at 95° for a few minutes. The cooled and filtered solution (at pH 7) was divided into two equal parts: to one was added an equal volume of distilled water and to the other an equal volume of 0·4% acetic acid. Each was heated at 100° for 30 min. *Found:* water solution, 5 units/ml.; 0·2% acetic acid solution, 20 units/ml. The heated 1 : 4000 solution in water was then divided into two equal parts, which were diluted with 0·5 vol. of water and 0·4% acetic acid respectively. Both were heated at 100° for 90 min. *Found:* water solution, less than 1 unit/ml.; 0·13% acetic acid solution, 5 units/ml.

This shows that a solution, partially inactivated at pH 7, cannot be reactivated by bringing to pH 3 and then heating at 100° (cf. Harington & Scott, 1929, for comparable results with the heat inactivation of solutions of crystalline insulin). Solutions of diplococcin inactivated by heat at pH 7 still give the protein reactions. Diplococcin is stable in glucose-broth medium at pH 7 for at least a week at 37°.

*Range of anti-bacterial effect of diplococcin*

The best preparation of diplococcin had a marked but transient effect on the growth of an authentic strain of *Streptococcus cremoris* at 24° when introduced into 2% glucose-broth at a concentration of 1 part in 2 millions, and a permanent bactericidal effect at 1 part in 200,000 when a small inoculum was used, e.g. 5000 viable bacteria/ml. Almost the full effect could be obtained in heart broth containing no added carbohydrate, and since growth here was poor in the absence of diplococcin, no dilution of the culture for sowing was necessary. The effect on a distinctly more vigorous strain of *Str. lactis* was only one-twentieth of the above. Diplococcin had also a definite transient inhibitory effect on *Staphylococcus aureus* (strains N.C.T.C. nos. 3095 and 3750) in 2% glucose-broth, at 37°, at a concentration of 1 part in 50,000, and a permanent bactericidal effect at 1 in 10,000, but only if an inoculum as small as one-hundredth of a loopful of a 1000-fold diluted culture was used per 5 ml. tube. The substance had a marked inhibiting effect, at a concentration of 1 in 20,000, upon a haemolytic streptococcus which grew vigorously in blood broth at 37°. It had no action on *Esch. coli* (as Whitehead found) no matter how highly diluted the culture for sowing might be.

## DISCUSSION

As Whitehead pointed out, it is not often that an organism militates against growth of other strains of its own species. There would seem to be more than one instance of this behaviour among the lactic streptococci, since one of Whitehead's inhibiting organisms (9S), unlike the two strains here

studied, did not ferment sucrose. It is more remarkable still that of all the Gram-positive cocci inhibited by diplococcin the most susceptible is an organism (*Str. cremoris*) that is practically indistinguishable from those which produce the inhibitor (cf. Meanwell, 1943, p. 21). It is therefore of some significance that the method used for the isolation of diplococcin from the bacterial cell is very similar to those employed by Lancefield (1928, 1933) and Stamp & Hendry (1937) for the preparation of protein antigens of haemolytic streptococci. Diplococcin may possibly be a component of the somatic antigen of the streptococci from which it is obtained, and it would be of interest to find out, by serological studies, if strains F7 and F8 contain antigenic constituents in common with *Str. cremoris*.

Nothing chemically distinctive concerning diplococcin has so far been noted, except perhaps the complete absence of sulphur. It is obviously an anti-bacterial substance in the same category as the sulphur-free polypeptides gramicidin and tyrocidine, the amino-acid composition of which has recently been worked out (Gordon, Martin & Synge, 1943 *a*, *b*). Unlike these polypeptides, diplococcin contains arginine residues, is insoluble in ethanol, shows no tendency to crystallize and is obviously of greater molecular complexity.


## SUMMARY

1. Two strains of milk streptococci which inhibit cheese starters produce apparently the same anti-bacterial substance, tentatively called diplococcin, which powerfully inhibits the growth of *Streptococcus cremoris*, a related organism. Other Gram-positive cocci are inhibited to a lesser extent.

2. Diplococcin occurs attached to the bacterial cells and also in the medium if the latter becomes markedly acidic as the result of carbohydrate fermentation.

3. Diplococcin is insoluble in absolute ethanol, is water-soluble, and appears to be a protein-like substance of relatively small molecular size. It contains no sulphur or phosphorus.

4. Diplococcin is stable at pH 4 at 100°, but not at pH 6–7.

5. A simple method is described for the isolation of diplococcin from bacterial cells grown in a semi-synthetic medium, the yield being about 20 mg./g. of dried cells separated from 5 l. of medium.


The author is greatly indebted to Prof. H. Raistrick, F.R.S., for suggesting this problem, to Mr L. J. Meanwell of United Dairies Research Laboratories for cultures and for information concerning them, and to Mr A. Edwards, without whose aid in the preparation of media this work would hardly have been possible.

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# Studies on Tannin Compounds

## 1. CHANGES DURING AUTOLYSIS OF MINCED CACAO BEAN

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It has long been considered that tannins and tannin-like compounds have an important influence on taste and flavour of cocoa and chocolate, and many tannin analyses have been reported. Unfortunately most of the methods of tannin estimation lack specificity and the position is further complicated by the fact that several closely allied substances may be present. The present paper provides evidence that several tannin-like components occur in the cacao bean and suggests a method of differentiating them.

Freshly gathered cacao beans are subjected to a process of fermentation. This process is, briefly, as follows: the beans, still enclosed in a white mucilaginous pulp, are heaped together in a wooden box where they remain for several days, being turned over or transferred to another box during this time. The beans are then sun-dried. During the fermen-

tation a peak temperature of about 50° is reached after approximately 72 hr. A full account of cacao fermentation may be found in Knapp (1937). The constituents of the bean undergo certain changes which are in part due to the natural enzymes and in part to various yeasts, moulds and bacteria. The fermentation results in reduction of the amount of tannin, besides other changes which are said to improve the flavour. In an attempt to separate the changes due to the enzymes of the bean and the enzymes of the micro-organisms, experiments were performed in which fresh cacao beans were finely minced and allowed to autolyze and the changes in tannin components followed at successive intervals of time.

## EXPERIMENTAL

### A. *Autolysis in air*

The testas were removed from about 500 g. fresh cacao beans and minced as rapidly as possible (about 2 min.). Zero time was taken as half-way through mincing, and at intervals a 25 g. portion of this material was added to 100 ml. of boiling water in a beaker. The contents were rapidly brought to the boil in order to inactivate the enzymes, and then left to cool. A representative sample of the intact, unkilled bean was prepared by plunging fresh beans with their testas into boiling water for 2 min., removing the testas, mincing and treating in the manner o the other samples. Acetone was added to each beaker to make the concentration 40% (by vol.) and the whole was left to stand overnight at room temperature (*c.* 26°) to extract the tannins. The boiling, in addition to the extraction with 40% acetone in the cold, was calculated to give maximum tannin extraction (Huber, 1929). Next day each preparation was filtered, washed with 40% acetone and made up to volume with 40% acetone. Measured fractions of these solutions were used for the following determinations based on the procedure proposed by Duthie (1938). (1) Tannin precipitated by saturated cinchonine sulphate solution; (2) a Stiasny determination on the filtrate from the cinchonine tannate ('residual Stiasny'); (3) a Stiasny determination on the original solution ('total Stiasny'). In addition, the amount of tannin was determined by a modified Lowenthal procedure (Association of Official Agricultural Chemists, 1940). The cinchonine tannate value was multiplied by 0·534 to convert to tannin (Jensen, 1928). The values obtained by the permanganate oxidation were expressed in terms of gallotannin for the sake of a basis of comparison.

In all experiments with this procedure the behaviour was closely similar, and an example is shown in Fig. 1. There was a rapid decrease with time in the amounts of cinchonine sulphate precipitate, residual Stiasny precipitate, total Stiasny precipitate and substances oxidized by permanganate. The values from each method of measurement reached constancy after approximately 50 min. and in no case was a zero value attained.

Duthie (1938) suggested that the residual Stiasny is a measure of 'catechin and similar phenolic compounds'. It will be noticed from Fig. 1 that the residual Stiasny fraction closely follows the downward trend of tannin precipitated by cinchonine sulphate and the total Stiasny fraction. It seems unlikely, therefore, that residual Stiasny

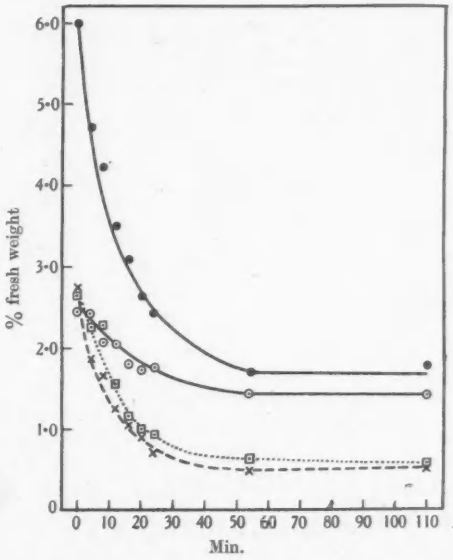


Fig. 1. The decrease with time of tannin fractions in minced cacao bean. ●——● Total Stiasny precipitate. ⊙——⊙ Cinchonine sulphate precipitate. ×——× Residual Stiasny precipitate. ⊡·····⊡ Tannin oxidized by permanganate.

measures catechin, which would be expected to remain constant during autolysis. Moreover, the catechin of the cacao bean appears not to be readily soluble. Adam (1928) found that up to 16 days' extraction with ether was necessary to remove all traces of catechin from dry cacao material free from fat and xanthine bases, and using the colorimetric method of Mitchell (1923), he estimated that unfermented beans contained about 0·8% of catechin, expressed on a dry weight basis, or approximately 0·4% on the fresh weight. It is unlikely that any catechin was extracted by the procedure adopted above, although Nierenstein (quoted in Jensen, 1928) mentions the possibility that catechin is converted to tannin during boiling. The residual Stiasny values of 2–3% of the fresh weight of the bean are much higher than would be expected if measuring catechin. No trace of catechin could be detected in the 40% acetone extract and it is concluded that catechin need not be taken into account in discussing the results obtained. The residual Stiasny

precipitate appears to be a measure of an integral part of the tannin molecule.

In some experiments the total material soluble in 40% (v/v) acetone at successive intervals was also measured. Two examples of the results obtained are shown in Table 1. There was a progressive decrease in the total amount of acetone-water soluble material and there was a high, fully significant, positive correlation between the amount of soluble material and the corresponding total Stiasny value. The linear regression coefficients of total Stiasny value (percentage fresh weight) on acetone-water soluble material (percentage fresh weight) ranged from 1·13 to 1·22 in different experiments, indicating that the decrease in total acetone-water soluble material was less than the decrease in total Stiasny value, from which it is inferred that there is a progressive, linear accumulation of some substance or substances during autolysis, soluble in 40% acetone. The nature of this was not determined. The amount of tannin, as measured by the cinchonine sulphate precipitate, the residual Stiasny precipitate and the total Stiasny precipitate, reached a constant value after the same length of time (approximately 54 min.). It appears that when minced cacao bean material is allowed to autolyze in the manner described above, a certain tannin fraction, subsequently referred to as 'oxidizable tannin', is rapidly transformed. The remaining fraction ('resistant tannin'), which also reacts with cinchonine sulphate and the Stiasny reagent, and has a positive value even after 110 min. hydrolysis (Fig. 1), is apparently not acted upon by the enzyme system responsible for the transformation of the oxidizable tannin. If

Table 1. *The amount of total Stiasny precipitate and the amount of material soluble in* 40% (v/v) *acetone in two samples of cacao beans at various times during autolysis*

| Time (min.) | Total Stiasny precipitate (% fresh weight) | Material soluble in 40% (v/v) acetone (% fresh weight) | Total Stiasny precipitate (% fresh weight) | Material soluble in 40% (v/v) acetone (% fresh weight) |
|---|---|---|---|---|
| 0 | 6·00 | 9·83 | 8·39 | 12·71 |
| 4 | 4·70 | 10·26 | 5·00 | 10·05 |
| 8 | 4·23 | 9·16 | — | — |
| 12 | 3·51 | 9·44 | — | — |
| 16 | 3·10 | 9·39 | 3·28 | 8·53 |
| 20 | 2·63 | 7·98 | — | — |
| 24 | 2·41 | 8·00 | — | — |
| 54 | 1·72 | 7·48 | 2·32 | 7·64 |
| 110 | 1·78 | 7·19 | — | — |
| | $r = +0·876$ (significant at $P = 0·01$); regression of total Stiasny on acetone-water soluble = 1·133 | | $r = +0·999$ (significant at $P = 0·01$); regression of total Stiasny on acetone-water soluble = 1·201 | |

*C*, *T* and *R* are the total amounts of tannin as represented by the cinchonine sulphate, total Stiasny and residual Stiasny determinations at any given time, and *c*, *t* and *r* the corresponding amounts after 54 min. (resistant tannin), then $C-c$, $T-t$ and $R-r$ will represent the amounts of oxidizable tannin at any given time. In Fig. 2, data for the oxidizable fractions alone have been plotted, viz. the residual Stiasny values ($R-r$), the difference between the total Stiasny and the residual Stiasny values, i.e. $[(T-t)-(R-r)]$, at successive times. Results are presented for five experiments with different samples of beans. It is seen that these two fractions bear a fairly close similarity to one another both in magnitude and trend. In all experiments with pigmented beans, consideration of the oxidizable tannin alone yielded results similar to those shown in Fig. 2, although the amounts of resistant tannin differed considerably. It thus appears that the tannin molecule is partitioned into two approximately equal portions by the action of cinchonine sulphate. The portion of the oxidizable tannin precipitated by cinchonine sulphate ($C-c$) does not always follow the total Stiasny minus residual Stiasny curve $[(T-t)-(R-r)]$ very closely (Fig. 2), and it is suggested that this is because the amount of cinchonine sulphate precipitate is dependent upon the pH (Ware & Smith, 1933), but the partition of the tannin molecule by the cinchonine sulphate is not so dependent on pH. Hence it is likely that the difference between total Stiasny and residual Stiasny is a better estimate of the tannin precipitated by cinchonine sulphate than the value calculated from the weight of cinchonine sulphate precipitate. In these experiments no attempt was made to control the pH before precipitation by cinchonine sulphate, although 150 ml. of saturated cinchonine sulphate, added to only 25 ml. of tannin solution, maintains the pH of precipitation within certain limits. If, as suggested, precipitation by cinchonine sulphate measures only approximately one-half of the tannin, the values obtained by the cinchonine sulphate method hitherto reported in the literature seriously underestimate the amount of tannin present in the cacao bean.

The fact that the main tannin component of the cacao bean quickly disappears during autolysis in air provides a simple means of estimating this fraction; it is only necessary to measure the decrease in tannin during 1 hr. autolysis. The results presented in Fig. 2 show that the amount of this tannin varies in different samples of beans.

### B. *Autolysis in distilled water*

A series of experiments was performed in which fresh, minced cacao beans were allowed to autolyze in distilled water. Several 250 ml. beakers of approximately similar weight were counterpoised in turn on a balance and water

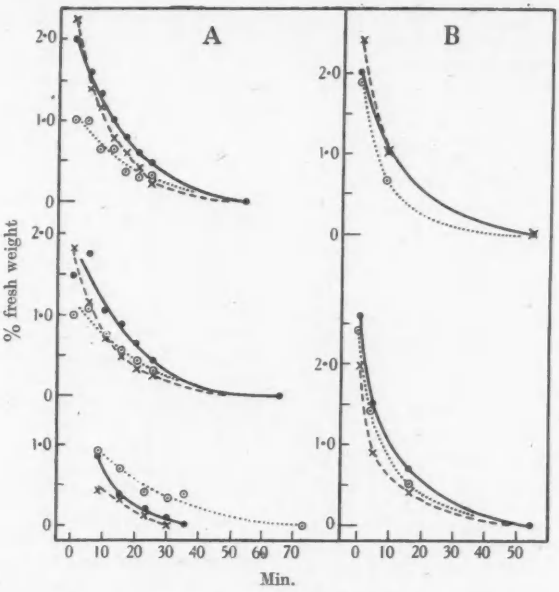


Fig. 2. The decrease with time of three fractions of the oxidizable tannin of minced cacao bean. ●——● Total Stiasny precipitate minus residual Stiasny precipitate. × ---- × Residual Stiasny precipitate. ⊙····⊙ Cinchonine sulphate precipitate.

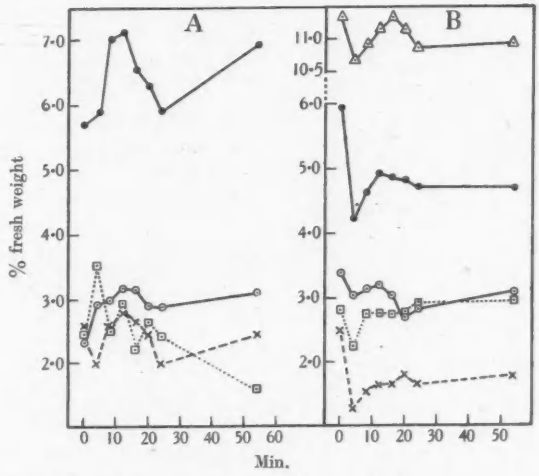


Fig. 3. The changes with time of tannin fractions in minced cacao bean standing under water. △——△ Total material soluble in 40% acetone. Other symbols as in Fig. 1.

added to bring the weight to 130 g., so that each beaker contained approximately 60 ml. water. Fresh beans with testas removed were quickly minced and 5 teaspoonfuls of the mince put in each beaker, which was then weighed. Boiling water was added to each beaker after a given time and the contents brought to the boil. The subsequent procedure was similar to that for the air autolysis experiments.

In these experiments there was no rapid decrease in tannin with time, presumably because of the restricted oxygen supply, but the amount of tannin increased. There was an initial fall in some cases before the increase occurred. Fig. 3A illustrates the results obtained in an experiment where no initial fall in tannin amount was observed, except in the case of the residual Stiasny at 4 min. Apart from this anomaly the cinchonine sulphate, total Stiasny and residual Stiasny fractions show similar trends. The maximum rise in each occurred after 12 min., except the values obtained by the permanganate method, where the maximum occurred after 4 min. The results were more variable by this last method than in the other procedures, probably owing to the difficulty in judging the end-point of the titration. In Fig. 3B is shown an example of the results found in some experiments where there was an initial drop and a subsequent rise in the values obtained by all four methods of measurement. The permanganate oxidation value slightly exceeded the initial value after 54 min., but in no other case did the subsequent values exceed the initial values. As in the previous experiment the maxima of the total Stiasny and cinchonine sulphate values were attained after 12 min. The maximum of the residual Stiasny values was reached after 20 min. The acetone-water soluble material of the successive samples showed a similar trend to the tannin fractions and reached a maximum, a little less than the initial value, after 16 min. It thus appears that in these experiments where oxygen was partially excluded from the minced beans, the amount of tannin increases over and above the amount initially present. The extra amount of tannin obtained in this way varied considerably with different samples of beans. It seems fairly certain that the amount of extra tannin recorded does not necessarily measure the true increase, since ordinary autolysis proceeds at the same time, both in water and for a short period before the minced bean is put in water, although every effort was made to reduce this time to a minimum. In other words the data obtained in water autolysis experiments are resultants of two opposite processes, disappearance of tannin due to enzyme oxidation and production of additional tannin from some precursor. As the first process is considerably slowed by lack of available oxygen, the second process is more prominent, resulting in a net increase of tannin. The fact that acetone-water soluble material shows a trend similar to that of tannin in these experiments suggests that the extra tannin is produced from a substance which is itself insoluble in 40% (v/v) acetone.

Owing to scarcity of cinchonine sulphate it was not possible to develop these experiments, but it is hoped to continue them in the future.

## DISCUSSION

The main tannin component of the cacao bean appears to be split into two approximately equal portions by cinchonine sulphate. In this connexion the data of Clarke, Frey & Hyland (1939) are of interest. They determined the increase in tannin in leaves of *Lespedeza sericea* during growth. A re-examination of their data shows that the amount of tannin fixed by hide-powder (revised Wilson-Kern method) is practically equal to the difference between the formaldehyde precipitate and fixable tannin during a $2\frac{1}{2}$-fold increase in tannin. Humphries (1943) pointed out that the residual Stiasny of the cacao bean probably measures the same substance as is fixed by gelatin (Lowenthal method), and it is probable that this portion is also fixed by hide-powder. Hence the tannin fraction in *Lespedeza* leaves measured by the formaldehyde precipitate minus fixable tannin in all likelihood corresponds with the fraction total Stiasny minus residual Stiasny in the cacao bean. If this is the case, the tannin of *Lespedeza* leaves appears to be of the same nature as that present in the cacao bean.

It was found that the amount of resistant tannin varied with different samples of beans. The ratio of the cinchonine tannin to the residual Stiasny value in the resistant tannin was by no means constant in different samples (Table 2) and this may mean

Table 2. *The amount of cinchonine tannin, total Stiasny precipitate and residual Stiasny precipitate present in the resistant tannin of different samples of cacao beans*

| Sample | Cinchonine tannin (% fresh weight) | Total Stiasny precipitate (% fresh weight) | Residual Stiasny precipitate (% fresh weight) | Ratio: Cinchonine tannin/ Residual Stiasny ppt. |
|---|---|---|---|---|
| *a* | 0·98 | 1·74 | 0·75 | 1·3 |
| *b* | 1·26 | 1·14 | 0·23 | 5·5 |
| *c* | 1·43 | 1·72 | 0·49 | 2·9 |
| *d* | 1·69 | 2·60 | 0·97 | 1·7 |
| *e* | 1·43 | 1·17 | 0·20 | 7·2 |
| *f* | 1·31 | 1·64 | 0·37 | 3·5 |

that at least two different tannin-like substances are present in the resistant tannin fraction. There was an indication that the amount of resistant tannin was small in unpigmented beans, but the large variety of cacao beans available have not been investigated sufficiently to be certain of the point.

Experiments were performed to see if any change takes place in the anthocyanin pigment, cyanin-3-monoside (Laurence, Price, Robinson & Robinson, 1938), during autolysis. Samples were exhaustively extracted with 90% (v/v) ethanol after various periods of autolysis, and measured portions boiled down *in vacuo* to remove ethanol, and treated with basic lead acetate. The lead precipitate was hydrolyzed for 1 hr. with N-HCl, and the amount of reducing sugar estimated. There was no significant change with time in the amount of reducing sugar (presumably mainly from the anthocyanin), which was taken as an indication that little change had taken place in the anthocyanin during autolysis. It is likely that, during the more drastic commercial fermentation, considerable changes in the anthocyanin do occur, which may lead to the development of tannin-like substances. Knapp & Hearne (1939) have reported the presence of leuco-anthocyanins in all parts of the cacao fruit, and these may be concerned in the residual tannin fraction.

The presence of *l*-epicatechin, closely related to cyanidin, in the cacao bean has been reported (Freudenberg, Cox & Braun, 1932). This may be the tannin-precursor found in the present investigation but so far direct evidence is lacking. The fact that a tannin-precursor is present is important, however, as the foregoing results indicate that unless the conditions during a commercial fermentation are mainly aerobic an increase in tannin, instead of the desirable decrease, may actually occur.

## SUMMARY

1. When fresh cacao beans are minced and allowed to autolyze, there is a rapid decrease in the amount of tannin as measured by (*a*) precipitation with cinchonine sulphate, (*b*) total Stiasny precipitate, (*c*) residual Stiasny precipitate, (*d*) oxidation by permanganate (Lowenthal method).

2. A constant value was attained after about 54 min. by all these methods and it is suggested that autolysis divides the tannin compounds into two fractions—oxidizable and resistant.

3. The oxidizable tannin appears to be split into two approximately equal portions by cinchonine sulphate.

4. Evidence for the existence of a tannin-precursor was obtained by allowing minced beans to autolyze in water, when an increase in tannin occurred. The tannin-precursor is not soluble in 40% (v/v) acetone.

5. The resistant tannin fraction appears to consist of at least two components.

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# Biochemical Characterization of the Actions of Chemotherapeutic Agents

## 2. A REACTION OF HAEMOLYTIC STREPTOCOCCI, INVOLVING PANTOTHENATE-USAGE, INHIBITED BY PANTOYLTAURINE, AND ASSOCIATED WITH CARBOHYDRATE METABOLISM

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The basis upon which pantoyltaurine was designed as an antibiotic agent (Snell, 1941; Kuhn, Wieland & Möller, 1941; McIlwain, 1942*a*; cf. Fildes, 1940), and subsequent investigations (McIlwain, 1944), suggested that the compound acted upon bacteria by affecting metabolic processes involving pantothenate. It is also suspected that other chemotherapeutic agents have their primary action upon

the metabolism of parasites, but in no case have the processes concerned been specified in detail. Such characterization could be expected to be of value in understanding the peculiarities and limitations of existing chemotherapeuticals and in suggesting new ones. This characterization has now been attempted in the case of pantoyltaurine.

During exploratory experiments the observation was made that the pantothenate concentration of streptococcal cultures normally diminished during their growth, but that this decrease was smaller in the absence of excess glucose or in the presence of pantoyltaurine. The disappearance of pantothenate was therefore followed in streptococcal suspensions containing various constituents of the growth media. Glucose catabolism was found to be associated with the process, and the acid formed during this breakdown was measured. The reaction in most experiments occupied a few hours at 37°; the streptococci used were grown with defined quantities of pantothenate, and the pantothenate of their cells was also taken into account. The concentrations of pantothenate and pantoyltaurine used in metabolic and growth experiments included those found *in vivo* during successful chemotherapy with the compound (McIlwain & Hawking, 1943).

## EXPERIMENTAL

The streptococcal cultures, the conditions of their maintenance, and the method of enumerating viable organisms, were those described in Part I (McIlwain, 1944). A Group D strain was also used; all were freshly passaged $\beta$-haemolytic streptococci. The reagents were those of Part I, except in the cases specified below.

*Casein hydrolysate prepared by acid in the presence of titanous salts.* The casein hydrolysate previously described contributed extraneous material to the harvested organisms and the following method of hydrolysis was chosen to avoid the 'humin' formation associated with the former preparation. The method was based on the findings of Sullivan & Hess (1937) (cf. also Lieben, 1943); the product is preferable for most bacteriological purposes in yielding a light-coloured medium which gives no precipitate on keeping or on making acid.

A mixture of concentrated hydrochloric acid (170 ml.) and technical 15% (w/v) titanous chloride (80 ml.) was added to casein (200 g.) in a conical beaker, mixed quickly before the liquid could be unevenly absorbed by the solid, and autoclaved for 45 min. at 120°. The solution was cooled in running water and taken to pH 6–6·5 with 40% NaOH (*c.* 240 ml.). A bulky, dark-coloured precipitate, mainly of titanium salts, separated. A small sample of the whole was boiled, filtered and tested by the addition of further small quantities of NaOH and HCl, and boiling; if a further precipitate formed, the bulk was adjusted in the sense suggested and a further specimen tested. The bulk, under conditions optimal for precipitation, was then boiled (frothing occurred initially and the precipitate lightened in colour) for 10 min., kept cool for 3 hr., and filtered through paper pulp on a Buchner funnel. Much of the phosphate of the casein was removed during precipitation.

*Basal medium.* A stock solution was prepared from the above filtrate by adding $KH_2PO_4$ (45 g.), oxalic acid (1 g.), NaOH to pH 7·6, water to 1000 ml., boiling gently for 5 min., keeping cold for at least 2 hr., and filtering through fluted papers. The reagents appeared to remove material contributed by the titanium solution as well as the calcium of the casein; the solution was kept with chloroform in the dark. The basal medium was prepared from it as required, by diluting 100 ml. to 700 ml., distributing in 70 ml. portions in plugged 250 ml. conical flasks, and autoclaving to sterilize and remove chloroform.

*Growth medium C.* The following solutions were mixed and made aseptically to a final volume of 100 ml. with M/18 phosphate buffer: basal medium (70 ml.), M-glucose (5 ml.), M/50 arginine and M/50 glutamine (2 ml.), Group *A* of McIlwain (1944) (5 ml.), M/2 $NaHCO_3$ (7 ml.), pantothenate and inoculum.

*Growth.* Freshly prepared medium C (100 ml.) was inoculated with approximately $10^6$ organisms from approx. 24 hr. old serum-broth-agar cultures of the streptococci, and incubated at 37° in 5% $CO_2$-air. Unless otherwise described (cf. Table 2), incubation was continued for 45–48 hr. and the culture neutralized at 18, 24 and (if necessary) at 42 hr. For this purpose, 1 ml. was taken aseptically and titrated with N/20 NaOH to pH 7·6; the necessary volume of N-NaOH was then added to the bulk. The additions necessary were usually of 4–5 ml. and the total alkali added per flask about 10 ml.

After growth, the dry weight of organisms in 10 ml. of the culture (or pooled cultures) was determined after washing three times with water and drying at 100°. The bulk of the organisms was collected by centrifuging and washed three times with M/18 phosphate buffer of pH 7·6 (10 ml./100 ml. culture) or, when noted, with saline.

*Reaction with pantothenate.* The majority of the experiments were carried out in centrifuge tubes which contained in a volume of 5 ml.: (1) a suspension of streptococci, usually of 10–50 mg. dry weight, (2) phosphate buffer, commonly M/15 of pH 7·6, (3) the casein hydrolysate of McIlwain (1939) at a final concentration of 2%, (4) M/20 glucose, (5) $2 \times 10^{-6}$M-pantothenate, (6) pantoyltaurine or other inhibitors. The tubes, loosely stoppered, were incubated in air at 37°, usually for 4 hr. with occasional shaking. The bacteria were separated by centrifuging and the solutions titrated to their original pH with N/5 NaOH, made to a standard volume, and sterilized for assay by autoclaving at pH 6·5–7. The organisms remaining in the centrifuge tube were washed twice with buffer, pH 7·6, and kept for pantothenate assay.

In the majority of the present experiments no attempt was made to control the change in pH brought about by the organisms, as it was used for estimating their acid formation and the conditions were chosen to simulate those of the cultures in which disappearance of pantothenate was first observed. The final pH approached 5·3; pantothenate disappearance continued at pH 5 in the presence of the organisms, but not in their absence nor in the presence of certain inhibitors. It also occurred when the solution was maintained at pH 7·6 throughout the reaction.

*Pantothenate assay.* Microbiological methods, using the growth of *Proteus morganii* (N.C.T.C. no. 2818) and an occasional comparison using the Group G streptococcus, were employed (McIlwain & Hawking, 1943). The reference tubes contained $10^{-8}$ to $10^{-9}$M-pantothenate. The quan-

tities of pantoyltaurine used in the present experiments did not interfere with the assay of the coincident pantothenate by *Pr. morganii*; when relatively high concentrations of streptococcal extracts were assayed, control experiments were carried out with addition of pantothenate to detect any inhibitory effect upon the growth of the test organism. Some values were confirmed by a photometric method to be described later.

*Pantothenate liberation from organisms* (Table 1)

Various methods of releasing pantothenate were carried out as follows. *Autoclaving* was at 120° for 20 min. *Autolyses* were carried out by suspending the washed organisms at different concentrations in buffers of varying pH, with a drop of benzene, and incubating at 37° for 1–3 days. *Digestion*: this was the procedure normally carried out and the conditions were based upon those of Cheldelin, Eppright, Snell & Guirard (1942) for the liberation of pantothenate from various materials. The washed bacteria (of about 30 mg. dry weight) in centrifuge tubes were suspended in M/10 acetate buffer, pH 4·7 (1 ml.); 2 % takadiastase (0·3 ml.) and 0·7 % purified papain (0·3 ml.), also in acetate buffer, were added and the whole incubated with a drop of benzene, at 37°, for 2 days, with occasional shaking. The solutions were then diluted, taken to pH 6·5–7 with NaOH, made to 5 ml., the tubes plugged and autoclaved, centrifuged, and the supernatant decanted sterilely for assay.

Two commercial specimens of takadiastase were found to contain satisfactorily small quantities of pantothenate (< 2 mμ mol./g.) but it was found necessary to purify the papain. This was done by ethanol precipitation according to Hellerman & Perkins's (1934) method, which reduced the pantothenate content of the enzyme from 50 to < 2 mμ mol./g. and doubled its gelatinase activity. The pantothenate of reagent controls in the normal digestion procedure described above was then less than could be assayed.

Table 1 shows that several procedures liberated approximately equal quantities of pantothenate from streptococci, those procedures using takadiastase tending to yield the highest quantities. Though the contribution of the enzymes to pantothenate liberation in the present experiments is doubtful, the enzymes have been included as a precaution (they increased the pantothenate obtained from certain tissues by Cheldelin *et al.* (1942)) and to render the results comparable with those of Thompson (1942). The present pantothenate-yields from streptococci are in fact notably lower than those recorded by Thompson for other bacterial species, but that this was not due to the technique employed was shown by examining certain of the organisms assayed by Thompson, when results comparable to his were obtained.

Benzene was added primarily to minimize contamination; in an experiment in which it was omitted, but which was not contaminated, the pantothenate liberation was no greater than in the presence of benzene. Benzene is known also to prevent the destruction of pantothenate by streptococci (see later); though there are further reasons (absence of glucose and casein) for the absence of this process during digestion, it was important to confirm that it did not occur, and this was done as follows. A batch of organisms (184 mg. dry weight) was divided into three portions; to one was added 0·5 ml. water, to another 0·5 ml. of $10^{-6}$ M-pantothenate and, to the last, 0·5 ml. of $10^{-5}$ M-pantothenate. All were digested and assayed by the standard procedures and the latter two found to contain both the native and added pantothenate.

Table 1. *Extraction of pantothenate from streptococcal cells*

| Process | Batch of organisms*; dry weight (mg.) | Pantothenate extracted | |
|---|---|---|---|
| | | mμ mol. | mμ mol./g. dry weight |
| *Standing* 24 hr. at 20° in 2 ml. phosphate buffer, pH 4·7 or 7·6 | I; 25 | 0·10 | 4 |
| *Autoclaving* at pH 4·7 or 7·0 | I; 25 | 0·35 | 14 |
| *Autolysis* 2 days at 37° in: | | | |
| (*a*) 5 ml. phosphate buffer, pH 7·6 | II; 24 | 0·30 | 12·5 |
| (*b*) 5 ml. phosphate, pH 4·7 | II; 24 | 0·40 | 17 |
| (*c*) 2 ml. acetate buffer, pH 4·7 | III; 17 | 0·43 | 25 |
| (*d*) 0·8 ml. acetate buffer, pH 4·7 | IV; 42 | 1·25 | 30 |
| *Digestion* 1 or 2 days at 37° in 2 ml. acetate buffer, pH 4·7: | | | |
| (*a*) alone or with papain | V; 50 | 1·4 | 28 |
| (*b*) with takadiastase | V; 50 | 1·75 | 35 |
| (*c*) with papain and takadiastase | V; 50 | 1·75 | 35 |
| | VI; 24 | 0·65 | 27 |
| | VII; 11 | 0·35 | 32 |

* Organisms: the Group G strain grown in medium C with pantothenate, $10^{-5}$ M. Details of processes are given in the text. The numbers used in designating batches of organisms in the different tables are independent and do not permit cross-reference.

*Attempted liberation of pantothenate from culture fluid after growth.* Portions of a culture fluid (100 ml.), in which 82 mg. dry weight of streptococci had been grown with 50 mμ mol. of pantothenate, were assayed after the following treatments: (i) filtration to sterilize; (ii) and (iii) autoclaving at pH 7·6 and 4·7 respectively; (iv) digestion at pH 4·7 with papain and takadiastase. In all cases, assay of the specimens gave values corresponding to $< 1$ mμ mol. of pantothenate in the whole of the culture fluid. The assays of culture fluids reported in the tables are after autoclaving at pH 7, without enzyme treatment.

## RESULTS

### A. *Disappearance of pantothenate from streptococcal cultures*

The disappearance of pantothenate was shown with the four strains of β-haemolytic streptococci (of Groups A, D and G) which were examined (Table 2). Disappearance of 50 mμ mol. of pantothenate from normal cultures of 100 ml. was almost complete. With increasing amounts of pantothenate, up to 1000 mμ mol./100 ml., the absolute amount disappearing increased; but so also did the proportion remaining. The pantothenate recovered from the organisms was in all cases small. Table 2 contains many results obtained during the growth of organisms for later experiments and illustrates the reasons for choosing the following conditions of growth in the majority of cases. (*a*) The cultures were neutralized during growth, since the yield of organisms was then nearly doubled. (*b*) Growth with the smaller quantity of pantothenate (50, not 1000 mμ mol./100 ml.) was preferred as the yield of organisms was the same as with the larger quantity, but less washing was required to separate them from pantothenate of their media. (*c*) Growth was continued until the second day as the yield of organisms increased, the pantothenate content of the media decreased and (see later) the majority of the organisms died.

In considering possible fates of the pantothenate, other than its metabolism by the organisms, the following points were examined: (*a*) *Stability of pantothenate during growth.* Previous observations by many authors suggested pantothenate to be stable under the temperature and pH conditions of the present experiments, and this was confirmed by incubating batches of media at pH 7·6 and at pH 5, which contained defined quantities of pantothenate. These were not found to be lost. (*b*) *Stability of pantothenate during sterilization of solutions.* Several solutions were assayed after autoclaving, or sterilizing-filtration, but no differences in their pantothenate contents were found. (*c*) *Stability of pantothenate of the organisms during digestion.* (*d*) *Absence of 'bound' pantothenate in the mother-liquors of growth.* Experiments, indicating that loss or apparent loss of the compound does not occur through these factors (*c*) and (*d*), have been described. (*e*) *Recovery of pantothenate from organisms.* The approximately equal results obtained under different conditions (the last five of Table 1) suggest the limit which is being approached to be a real one. Repetition of enzyme digestion yielded no further pantothenate. (*f*) *The buffer used in washing the organisms.* When tested separately this contained very little pantothenate.

Table 2. *Disappearance of pantothenate during growth of streptococci*

| Organism and conditions of growth | Pantothenate/culture of 100 ml. Added (mμ mol.) | After growth: In solution (mμ mol.) | After growth: In organism (mμ mol.) | After growth: Total; % of that added | Yield of organisms (dry wt.; mg./100 ml. of final culture) | Acid produced (m mol. NaOH needed/100 ml.) |
|---|---|---|---|---|---|---|
| Group A (Richards); culture neutralized and grown for 42 hr. | 50 | <1 | 0·5 | <3 | 52 | 8·9 |
| Group A (7188); neutralized; 67 hr. | 50 | <1 | 0·25 | <2·5 | 16 | 4·1 |
| Group D; neutralized; 67 hr. | 50 | 1 | 0·25 | 2·5 | 55 | 9·8 |
| Group G; neutralized; 18 hr. | 50 | 2 | 0·5 | 5 | 53 | 5·7 |
| Group G; neutralized; 42 hr. | 50 | <1 | 0·3 | <2·6 | 80 | 9·7 |
| Group G; neutralized; 45–48 hr. | 50 | <1 | 0·2–0·4 | <2·8 | Mean of 17 exps.: 77·2 (Standard deviation, 11·6) | Mean of 17 exps.: 10·72 (Standard deviation, 1·15) |
| Group G; not neutralized; 42 hr. | 500 | 100 | 1·3 | 20 | 44 | — |
| Group G; neutralized; 42 hr. | 500 | 100 | 2·2 | 20 | 82 | — |
| Group G; neutralized; 45–48 hr. | 1000 | 300–500 | 1·3–2·4 | 30–50 | Mean of 9 exps.: 78·4 (Standard deviation, 10·9) | Mean of 9 exps.: 10·18 (Standard deviation, 1·65) |

The results of the remainder of this paper show that pantothenate disappearance is not a necessary concomitant of growth but can be influenced independently of many other processes then occurring.

### B. *Dissociation of pantothenate metabolism from streptococcal growth and viability*

The results of Table 2 demonstrate that the disappearance of pantothenate from streptococcal cultures is not proportional to the amount of growth; 77–78 mg. of the Group G organism removed from 49 to 500 or 700 mμ mol. of pantothenate, according to the amount initially present. Also, results of growth with larger quantities of pantothenate (not quoted here) tended to show that the growth of a culture could precede much of the pantothenate-disappearance. The effect of streptococci, collected and washed, upon pantothenate in the presence of other constituents of the cultures was therefore examined. Table 3 gives the results of experiments in which conditions were varied so that the pantothenate remaining after reaction under different circumstances (which will be discussed later) varied over a range of 150-fold. Change in dry weight of the organisms was not, however, detectable. Their pantothenate content, also, did not increase. The usage of pantothenate in several experiments of Table 3 is 10 mμ mol., which during growth of the organisms themselves corresponds to an increase in mass of 16 mg. dry weight; any increase during the experiments was, however, of the order of 1 mg. Pantothenate disappearance in the presence of streptococci is thus not dependent upon growth of the organisms.

The organisms used during the experiments of Table 3 were, however, viable in the sense that they could be subcultured from the suspensions after exposure. As the suspensions contained many millions of organisms, a closer examination was necessary in order to find whether the reaction depended upon the presence of viable organisms.

Table 3. *The disappearance of pantothenate in washed streptococcal suspensions; its independence of growth*

(Each tube contained hydrolyzed casein, phosphate, streptococci and the additional substances listed below in a final volume of 5 ml. Exposure was at 37° and for 4 hr. except in the case indicated (*), which was for 1 hr. The first five tubes contained equal amounts of Suspension I, and the last of Suspension II, of different batches of the Group G streptococcus grown in pantothenate, $5 \times 10^{-7}$ M.)

| Additional substances | | | After exposure | | |
|---|---|---|---|---|---|
| Pantothenate (mμ mol.) | Glucose (m mol.) | Inhibitors | Pantothenate in solution (mμ mol.) | Acid produced (m mol. NaOH to neutralize) | Dry weight of organisms (mg.) |
| | | Suspension I | | | |
| 0 | 0 | 0 | <0·05 | 0 | 43·8 |
| 10 | 0 | 0 | 7·25 | 0 | 44·0 |
| 10 | 0·25 | 0 | <0·10 | 0·106 | 44·8 |
| *10 | 0·25 | 0 | <0·15 | 0·070 | 45·1 |
| 10 | 0·25 | Pantoyltaurine, 20 μ mol. | 7·25 | 0·114 | 44·1 |
| | | Suspension II | | | |
| 0 | 0·25 | 0 | <0·07 | 0·118 | 35·2 |
| 10 | 0·25 | 0 | 1·25 | 0·116 | 35·2 |
| 10 | 0·25 | Phenol, 20 μ mol. | 6·5 | 0·064 | 36·9 |
| 10 | 0·25 | Propamidine, 1 μ mol. | 6·5 | 0·074 | 34·7 |
| 10 | 0·25 | Pantoyltaurine, 20 mμ mol. | 3·0 | 0·130 | 33·4 |

Table 4. *Glycolysis and the disappearance of pantothenate in streptococcal suspensions; their independence of viability*

(Each tube contained hydrolyzed casein, phosphate, glucose (0·25 m.mol.), pantothenate (10 mμ mol.) and preparations, in 5 ml., of the Group G organism (grown with pantothenate, $5 \times 10^{-7}$ M) described below, and was incubated for 4 hr. at 37°.)

| Organisms/tube | | | Pantothenate | | | Acid production | | |
|---|---|---|---|---|---|---|---|---|
| | | | Remaining in solution | Disappearance | | | | |
| Preparation | Dry weight (mg.) | Number of viable organisms | (mμ mol.) | mμ mol./ g. dry weight | mμ mol./ $10^6$ viable organisms | M/5 NaOH used (ml.) | m mol./ g. organisms | m mol./ $10^6$ viable organisms |
| Growth: 18 hr.; washed | 14 | $8{\cdot}3 \times 10^8$ | 0·5 | 680 | 1·1 | 0·52 | 7·5 | 0·013 |
| Growth: 18 hr.; washed | 3·5 | $2{\cdot}1 \times 10^8$ | 9·0 | 290 | 0·5 | 0·15 | 8·6 | 0·014 |
| Growth: 48 hr.; washed | 25 | $2{\cdot}3 \times 10^6$ | 0·3 | 390 | 420 | 0·69 | 5·5 | 6·0 |
| Growth: 48 hr.; washed | 8·3 | $7{\cdot}7 \times 10^5$ | 4·0 | 720 | 780 | 0·33 | 8·0 | 8·6 |
| Growth: 42 hr.; unwashed | 20 | $5{\cdot}7 \times 10^7$ | 1·3 | 440 | 15 | 0·49 | 4·9 | 0·17 |
| Growth: 42 hr.; washed | 20 | $6{\cdot}3 \times 10^7$ | 1·5 | 430 | 13·5 | 0·41 | 4·1 | 0·13 |

This was carried out by determining, in cultures grown and collected differently, both the reaction with pantothenate and the numbers of viable organisms. Table 4 compares the pantothenate disappearance of different suspensions with two properties. It shows that while the disappearance per viable organism varied over a range of more than 1000-fold, that per unit of dry weight varied only $2\frac{1}{2}$-fold. The latter variation, moreover, was not entirely a property of the suspension but also depended upon the mass of organisms used (i.e. upon the present assessing not using methods necessary to kinetic investigations).

It was estimated that the suspension collected 18 hr. after inoculation contained at least 70% of viable organisms, while that at 48 hr. contained about 0·1%. Growth for the longer period was adopted as the standard procedure in the following experiments.

pyruvate was substituted. A mixture of amino-acids replaced the casein hydrolysate, though it was less effective. With organisms which had been washed with saline instead of the phosphate usually employed, replacement of the phosphate in the normal reaction-mixture by bicarbonate did not decrease pantothenate metabolism. The casein hydrolysate, however, contributed a little phosphate to the medium. With the saline-washed organisms, pantothenate disappearance was slightly increased by addition of a magnesium salt. The reaction proceeded anaerobically as well as aerobically (Table 6). Although it is dissociated from growth, pantothenate disappearance increases with time (Table 6).

### D. *Effect of pantoyltaurine and other inhibitors upon pantothenate metabolism*

The present interest in the reaction of streptococci with pantothenate lies in its prevention by

Table 5. *Components necessary for pantothenate metabolism*

| Components (other than pantothenate) in 5 ml. | | | | | Pantothenate (mμ mol.) | | | |
|---|---|---|---|---|---|---|---|---|
| Glucose or surrogate (μ mol.) | Casein hydrolysate or mixed amino-acids* (mg.) | Phosphate (275μ mol.) or bicarbonate (250μ mol.); $MgSO_4$ (4μ mol.) | Dry weight of organisms (mg.) | Added | After growth | Disappearance/g. dry weight | Acid production (m mol. NaOH required to neutralize) |
| **Suspension I, washed with saline** | | | | | | | |
| 0 | Hydrolysate, 50 | Phosphate + $MgSO_4$ | 51 | 10 | 5–7 | 60–100 | 0 |
| Glucose, 250 | ,, ,, | ,, ,, | 51 | 10 | 0·25 | 190 | 0·202 |
| ,, ,, | ,, 5 | ,, ,, | 51 | 10 | 2·5 | 145 | 0·130 |
| ,, ,, | Amino-acids, 65 | ,, ,, | 51 | 10 | 2·0 | 155 | 0·120 |
| ,, ,, | Hydrolysate, 50 | Bicarbonate ,, | 51 | 10 | 0·15 | 195 | 0·20 |
| ,, ,, | ,, ,, | Phosphate only | 51 | 10 | 1·0 | 175 | 0·162 |
| **Suspension II, washed with phosphate buffer** | | | | | | | |
| Glucose, 250 | Hydrolysate, 50 | Phosphate only | 44 | 10 | <0·05 | >230 | 0·118 |
| ,, ,, | Amino-acids, 65 | ,, | 44 | 10 | 1·0 | 200 | 0·070 |
| ,, ,, | ,, ,, | ,, | 44 | 1 | <0·05 | > 20 | 0·074 |
| Pyruvate | ,, ,, | ,, | 44 | 10 | 6·5 | 80 | 0 |
| ,, | ,, ,, | ,, | 44 | 1 | 0·15 | 19 | 0 |
| **Suspension III, washed with phosphate buffer** | | | | | | | |
| Glucose, 250 | Hydrolysate, 50 | Phosphate only | 0 | 10 | 10 | 0 | 0 |
| ,, ,, | ,, ,, | ,, | 25 | 10 | 0·3 | 390 | 0·138 |
| Succinate, 500 | ,, ,, | ,, | 25 | 10 | 10 | 0 | 0 |
| Glycerol, 500 | ,, ,, | ,, | 25 | 10 | 10 | 0 | 0 |
| 0 | ,, ,, | ,, | 25 | 10 | 8·5 | 60 | 0 |

Time of exposure, 4 hr.

* The group of 14 amino-acids of Gladstone (1939), in the proportions there given.

### C. *Components necessary for metabolism of pantothenate by streptococcal suspensions*

The experiments of Table 5 showed that, of the constituents of the growth media in which disappearance of pantothenate was first observed, both glucose and the casein hydrolysate were necessary to pantothenate metabolism, though both could to some extent be replaced by related materials. Succinate and glycerol did not replace glucose, but disappearance of some pantothenate occurred when small quantities of pantoyltaurine. This has already been indicated in Table 3 and the effects with several different batches of organisms are given in Table 6. The phenomenon has been shown with 16 different batches of organisms, grown in the present media for periods varying from 1 to 3 days and with initial pantothenate concentrations of $5 \times 10^{-7}$ to $10^{-5}$ M. No batch has failed to show the phenomenon, and the majority, grown with $5 \times 10^{-7}$ M-pantothenate, showed the optimal effect recorded in Table 6 for suspension II. It therefore appears to be a charac-

teristic of the organisms, though the degree of their reaction with pantothenate is variable, as is shown in Table 6. The amount of pantothenate in the organisms was always small. The maximum effect—the disappearance of little of the 10 mμ mol. of pantothenate in the presence of pantoyltaurine, while controls showed the disappearance of 99% of the substrate—was usually given by 50 to 100 mμ mol. of pantoyltaurine (see Table 6). Lesser effects are given by smaller quantities, and these are such that the quantities of pantothenate and pantoyltaurine which interact in producing the metabolic effect are of the same order of magnitude. The magnitudes are especially close if, as in the growth of certain bacteria (Snell, 1941), *l*-pantoyltaurine only is effective; the pantoyltaurine used in the present experiments was racemic.

Tables 3 and 6 show, however, that inhibition of reaction with pantothenate is not in itself a characteristic property of pantoyltaurine. It is the major inhibitory effect, which has yet been recorded, of the compound upon a metabolic process (cf. McIlwain, 1944) and is not given by its products of hydrolysis (Table 6), but is shown by benzene, by high concentrations of phenol and malonate, and by relatively low concentrations of propamidine and

Table 6. *Effects of inhibitors, etc. upon pantothenate metabolism*

Standard conditions of exposure, except when stated in the fourth column. All reaction-mixtures except the first initially contained pantothenate, 10 mμ mol.

| Streptococci | | | Addenda/5 ml. | | Pantothenate (mμ mol.) remaining | | Acid produced (μ mol. NaOH to neutralize) |
|---|---|---|---|---|---|---|---|
| Batch no. | Initial pantothenate content of their growth media (M) | Dry weight (mg.) | Inhibitors (μ mol.); or other special feature | | Solution | Organisms | |
| I | $5 \times 10^{-7}$ | 54 | None; no pantothenate | | <0·05 | 0·05 | 62 |
| | | | None | | 2·0 | 0·10 | 62 |
| | | | Pantoyltaurine, | 5 | 8–10 | 0·18 | 58 |
| | | | ,, | 0·5 | 8–10 | 0·25 | 66 |
| | | | ,, | 0·005 | 8–10 | 0·20 | 66 |
| II | $5 \times 10^{-7}$ | 28 | None | | <0·05 | 0·20 | 150 |
| | | | Pantoyltaurine, | 0·5 | 10 | — | 148 |
| | | | ,, | 0·15 | 10 | — | 136 |
| | | | ,, | 0·05 | 10 | — | 160 |
| | | | ,, | 0·015 | 1·0 | 0·25 | 148 |
| III | $10^{-5}$ | 26 | None | | 2·0 | — | 104 |
| | | | Pantoyltaurine, | 2 | 10 | — | 70 |
| | | | ,, | 0·4 | 10 | — | 78 |
| | | | ,, | 0·08 | 5 | — | 90 |
| | | | ,, | 0·02 | 4 | — | 94 |
| | | | ,, | 0·004 | 2 | — | 102 |
| IV | $5 \times 10^{-7}$ | 60 | None | | 0·3 | 0·15 | 66 |
| | | | Pantoyltaurine, | 20 | 8·0 | 0·15 | 70 |
| | | | Hydrolyzed pantoyltaurine, | 20 | 0·5 | 0·15 | 72 |
| | | | Phenol, | 100 | 6·0 | 0·05 | 0 |
| | | | Salicylate (see text), | 100 | 0·2 | — | 82 |
| | | | Propamidine, | 4 | 2·0 | 0·20 | 50 |
| V | $5 \times 10^{-7}$ | 22 | None | | 1·2 | — | 122 |
| | | | Malonate, | 100 | 5·0 | — | 0 |
| | | | ,, | 10 | 1·1 | — | 120 |
| | | | Iodoacetate, | 20 | 9·0 | — | 0 |
| | | | ,, | 2 | 9·0 | — | 0 |
| | | | ,, | 0·5 | 2·0 | — | 100 |
| VI | $5 \times 10^{-7}$ | 54 | None | | <0·15 | 0·15 | 150 |
| | | | Sulphanilamide, | 10 | <0·15 | 0·20 | 142 |
| | | | Benzene (saturated) | | 6·0 | 0·15 | 52 |
| VII | $10^{-5}$ | 14 | None | | 0·25 | — | 58 |
| | | | Organisms heated 30 min. at 55° | | 8–10 | — | 0 |
| | | | Organisms heated 30 min. at 65° | | 10 | — | 0 |
| VIII | $5 \times 10^{-7}$ | 68 | Exposure: 4 hr. anaerobically | | <0·10 | — | 62 |
| | | | Exposure: 4 hr. aerobically | | <0·10 | 0·10 | 56 |
| | | | Exposure: 1 hr. aerobically | | 0·15 | 0·15 | — |
| | | | Exposure: ¼ hr. aerobically | | 3·8 | 0·10 | — |

iodoacetate. Salicylate and sulphanilamide are notable in having little effect at relatively high concentrations.

### E. *Relation of pantothenate metabolism to glycolysis*

During the course of these investigations it was found that the effect of pantoyltaurine upon pantothenate metabolism could be more closely defined by taking into account also the acid formation from glucose, which is associated with the disappearance of pantothenate from streptococcal cultures and suspensions. Such glycolysis is a notable property of the present organisms; Table 2 shows that it is a major reaction during growth, and Tables 3 and 5, that the omission of glucose from the reaction mixture from which streptococci normally caused pantothenate disappearance, prevented or greatly reduced its disappearance. Acid production during metabolic experiments was therefore determined and is recorded in Tables 2–6.

The following points show a certain, but significantly incomplete, relationship between the degree of glucose metabolism and of pantothenate disappearance under these conditions:

(1) Acid formation from glucose, like pantothenate-metabolism, is independent of growth of the organisms (Table 3) and of their viability (Table 4) but varies with the dry weight of organisms in roughly the same manner as their pantothenate-metabolism (Table 4).

(2) The casein hydrolysate gave maximum effects both in acid formation and pantothenate metabolism; aerobic conditions are not necessary to either process; magnesium salts slightly increase both; added phosphate is necessary to neither. Amino-acids in place of the casein hydrolysate allowed both processes to proceed at a slower rate; pyruvate metabolism is related to that of glucose and the compound permitted a slow reaction with pantothenate (Table 5).

(3) Inhibition of both processes under the conditions of Tables 3 and 6 is complete or nearly so with iodoacetate, 2–20 $\mu$ mol., but not with 0·5 $\mu$ mol. It is considerable with malonate, 100 $\mu$ mol.; phenol, 100 $\mu$ mol.; with benzene, and after heating the streptococci to 55 or 65°. Sulphanilamide, 10 $\mu$ mol., and the lower quantity of malonate, affected neither process. In the instance recorded, salicylate affected neither process but on other occasions it has been found to affect both.

The correlations which have been described are only semi-quantitatively expressed, as the present experiments do not measure the course of pantothenate and glucose metabolism. The findings, however, show a major difference between the behaviour of pantoyltaurine and of other inhibitors. Results with the first three suspensions of Table 6 indicate that although the compound may depress glycolysis, this has not been found to occur to an extent of greater than 33 % and may not occur at all even with maximal inhibition of pantothenate metabolism. The relatively small effect of pantoyltaurine upon the more accurately measured *course* of glycolysis was previously reported (McIlwain, 1944). The peculiarities of the metabolic effects of pantoyltaurine are thus its inhibition of pantothenate-disappearance when present in extremely small concentrations, and the fact that inhibition of the process, even when brought about by relatively high concentrations of pantoyltaurine, occurs without a corresponding inhibition of glycolysis.

## DISCUSSION

Previous findings have indicated that the need for pantothenate in streptococcal growth may be referred to its necessity to processes, probably enzymic, which are essential to growth of the organisms (McIlwain, 1942*b*). It is now suggested that the process by which pantothenate disappears from cultures, and by which it also disappears in the present metabolic experiments, may be one to which the actions of pantothenate and pantoyltaurine upon growth can be ascribed. This suggestion has been derived from the following considerations:

(1) The action of pantoyltaurine upon streptococcal growth is specifically connected with pantothenate (McIlwain, 1942*a*).

(2) Both growth and the metabolic process are very sensitive to pantoyltaurine, but of the two the metabolic process is the more sensitive. This order of sensitivity may not be entirely necessary to the causal relationship suggested above, but more satisfactory evidence for the relationship is afforded by it than if growth were to be the more sensitive. The order of sensitivity found is consistent with certain phenomena manifested in the course of growth (unpublished observations).

(3) Glycolysis, with which the metabolic process is associated, is a reaction especially important to the streptococci and pneumococci which are most sensitive to pantoyltaurine. Pantothenate has been found to be connected with carbohydrate metabolism in a variety of organisms: in *Pr. morganii* (Dorfman, Berkman & Koser, 1942; Hills, 1943), yeasts (Williams, Mosher & Rohrman, 1936; Teague & Williams, 1942) and animals (Wright, 1942). The latter investigation is of especial relevance to the present work, as it was found that administration of glucose to rabbits was followed by a lowering of their blood-pantothenate.

(4) Added pantothenate is, however, not necessary to glycolysis of the present organisms; Table 2 shows the reaction during growth to be unaffected by the presence of excess pantothenate in the culture

fluid, and Tables 3 and 6 that the glycolysis of suspensions is unaffected by the compound. A small stimulation only was found in previous experiments with the same organism (McIlwain, 1944) in which, in contrast with the present work, the course of glycolysis was measured. Pantothenate is always present in small quantities in the organisms; nevertheless, if free pantothenate is present, it is metabolized. As the association of the usage of added pantothenate with glycolysis is facultative, it is understandable that pantoyltaurine should be able, as is found, to depress pantothenate-usage without much effect upon glycolysis. The organism in presence of pantothenate and pantoyltaurine is then reverting to a condition similar to that obtaining when neither is present. This condition may be adequate for glycolysis but is not adequate for growth. The association of pantothenate-usage with growth is obligatory: growth is strictly limited by the quantity of pantothenate added when that is below a given concentration (McIlwain, 1944), and the limiting quantities are completely removed from the media. Pantoyltaurine in such cases prevents both the pantothenate metabolism and growth.

(5) To explain inhibition of growth by a process which preserves the growth-essential, pantothenate, it can be supposed that, in its functioning, pantothenate undergoes a cycle of changes, which is interrupted by pantoyltaurine, and of which at least one phase is relatively unstable in the presence of the reaction mixture.


## SUMMARY

1. Pantothenate disappeared from streptococcal cultures and from certain reaction mixtures containing pantothenate through a process for which the organisms were necessary but which was independent of their growth and viability.

2. This metabolism of pantothenate was associated with the presence of glucose and a casein hydrolysate (or related materials) and perhaps of magnesium, but was independent of oxygen supply.

3. When glycolysis was prevented by omitting or adding various substances, or by treating the organisms, pantothenate metabolism usually also ceased.

4. The presence of pantoyltaurine is exceptional among the circumstances investigated, in that it causes inhibition of pantothenate metabolism without a corresponding inhibition of glycolysis.

5. Streptococci normally contain pantothenate and are capable of glycolysis in the absence of added pantothenate, but if it be assumed that both glycolysis and the presence of excess pantothenate are needed for the formation of a substance essential to streptococcal growth, and that such formation is inhibited by pantoyltaurine, then it is understandable that, as observed, (i) absence of added pantothenate inhibits growth, (ii) inhibition of glycolysis stops pantothenate metabolism, (iii) pantoyltaurine stops pantothenate metabolism and growth, but not glycolysis.


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# A Quantitative Micro-method for the Separation of Inorganic Arsenite from Arsenate in Blood and Urine


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Studies on the metabolism of inorganic arsenic compounds in the animal body have been greatly hampered by the lack of a suitable quantitative method for the separation of trivalent from pentavalent inorganic arsenic in the body fluids. Precipitation methods for the separation of arsenite from arsenate in urine are unsatisfactory, as shown by Joachimoglu (1916), whose method was based on

the observation that, on electrolysis in strongly alkaline solution, arsine is evolved from arsenite but not from arsenate. However, as Joachimoglu recognized, the reduction of arsenite to arsine is not quantitative.

Klein & Vorhes (1939) described a method based on earlier work by Tarugi & Sorbini (1912), for the determination of total arsenic in biological materials, which involved the reduction of arsenate to arsenite and subsequent extraction of the latter from acid solution with sodium ethyl xanthate dissolved in carbon tetrachloride. Although the authors did not actually state that arsenic acid does not react with the xanthate reagent to form a carbon tetrachloride-soluble product, they implied that this is the case. In the present work it has been found possible to achieve a quantitative separation of arsenite from arsenate in blood and urine, freed of interfering substances, by extraction with the xanthate reagent, thereby allowing them to be separately estimated by any of the usual methods for total arsenic. This procedure seems to be more satisfactory than any that have hitherto been used.

## EXPERIMENTAL

*Preparation of the xanthate reagent* (Klein & Vorhes, 1939). NaOH pellets were added to pure dry ethanol in the proportion of 1 g. to 10 ml. The mixture was warmed and shaken vigorously for about 15 min., centrifuged and the supernatant liquid decanted off. The alkali content was determined by titration of a sample, and adjusted to a concentration of 7% (w/v). To 9 vol. of this solution, 1 vol. of redistilled carbon disulphide was added slowly with cooling, and, after mixing and filtering, the product was stored in the ice-box. A fresh solution was made fortnightly. The xanthate reagent was prepared as required by diluting 50 ml. of the alcoholic sodium ethyl xanthate to 1 l. with dry, redistilled carbon tetrachloride.

*Separation technique.* The arsenic-containing solution (80 ml. or less) was measured into a 150 ml. separating funnel and acidified with 10 ml. conc. $H_2SO_4$ diluted with 2–3 vol. of water. The solution was extracted by vigorous shaking for 2 min. with one 40 ml. and two 20 ml. portions of the xanthate reagent, 5 ml. of carbon tetrachloride being run through the funnels to wash down the stems after each extraction. The combined extracts were washed with one 10 ml. portion of 2 N-$H_2SO_4$ (this washing being added to the aqueous phase), and evaporated down after addition of water. Total arsenic was determined (Levvy, 1943) on the evaporated extract (arsenite fraction), and on the aqueous phase (arsenate fraction).

To avoid the extremely stable emulsions, which form with the xanthate reagent and blood or urine, the extractions were carried out on dialysates, or, in the case of blood, on trichloroacetic acid filtrates.

*'Blank' corrections.* Concurrently with each experiment, 'blank' solutions were extracted under identical experimental conditions. The corrections so obtained were applied to all results.

*Separation of arsenite from arsenate in simple solution.* Various amounts of arsenite and arsenate (as standard solutions of their sodium salts) were pipetted into volumetric flasks, and the solutions diluted to 100 ml. Total arsenic was determined on 40 ml. of each solution, and the remaining 60 ml. submitted to the xanthate procedure (Table 1).

*Separation of arsenite from arsenate added to blood dialysates.* 40 ml. of a haemolysate prepared from washed red blood cells were dialyzed in 'Visking' sausage casing against 80 ml. distilled water for 24 hr. in the ice-box. To this dialysate in a volumetric flask, known quantities of arsenite and arsenate were added, and the volume made up to 100 ml. Samples were analyzed for total arsenic, arsenite and arsenate, the results of four separate experiments being shown in Table 2.

Table 1. *Separation of arsenite from arsenate in simple solution*

| Total arsenic | | | Arsenite | | | Arsenate | | |
|---|---|---|---|---|---|---|---|---|
| Added (μg. As) | Found (μg. As) | Recovery (%) | Added (μg. As) | Found (μg. As) | Recovery (%) | Added (μg. As) | Found (μg. As) | Recovery (%) |
| 105·0 | 113·7 | 108 | 20·0 | 16·3 | 82 | 85·0 | 68·0 | 80 |
| 112·0 | 107·3 | 96 | 52·0 | 48·3 | 93 | 60·0 | 57·7 | 96 |
| 113·0 | 99·0 | 88 | 88·0 | 81·0 | 92 | 25·0 | 23·2 | 93 |
| 120·5 | 120·0 | 100 | 50·0 | 47·3 | 95 | 70·5 | 73·2 | 104 |
| 119·4 | 117·8 | 99 | 64·0 | 64·3 | 100 | 55·4 | 57·6 | 104 |
| 119·2 | 117·8 | 99 | 84·0 | 81·7 | 97 | 35·2 | 32·5 | 92 |
| 55·0 | 54·8 | 100 | 30·0 | 31·3 | 104 | 25·0 | 26·5 | 106 |
| 104·6 | 112·0 | 107 | 24·0 | 28·0 | 117 | 80·6 | 82·5 | 102 |
| 49·0 | 54·3 | 111 | 16·0 | 14·7 | 92 | 33·0 | 34·2 | 104 |
| 184·0 | 195·8 | 106 | 24·0 | 27·0 | 113 | 160·0 | 158·7 | 99 |

Table 2. *Separation of arsenite from arsenate added to blood dialysates*

| Total arsenic | | | Arsenite | | | Arsenate | | |
|---|---|---|---|---|---|---|---|---|
| Added (μg. As) | Found (μg. As) | Recovery (%) | Added (μg. As) | Found (μg. As) | Recovery (%) | Added (μg. As) | Found (μg. As) | Recovery (%) |
| 210·2 | 205·5 | 98 | 180·0 | 165·0 | 92 | 30·2 | 35·0 | 116 |
| 180·8 | 179·5 | 99 | 60·0 | 63·0 | 105 | 120·8 | 111·3 | 92 |
| 70·3 | 66·5 | 95 | 30·0 | 30·7 | 102 | 40·3 | 42·0 | 104 |
| 185·1 | 185·5 | 100 | 24·0 | 23·7 | 99 | 161·1 | 163·3 | 101 |

Table 3. *Separation of arsenite from arsenate added to blood haemolysates*

(Extraction procedure on aqueous dialysates)

| | Total arsenic | | | Arsenite | | | Arsenate | | |
|---|---|---|---|---|---|---|---|---|---|
| | Added (μg. As) | Found (μg. As) | Recovery (%) | Added (μg. As) | Found (μg. As) | Recovery (%) | Added (μg. As) | Found (μg. As) | Recovery (%) |
| 1 | 90·7 | 85·5 | 94 | 45·2 | 37·8 | 84 | 45·5 | 47·5 | 104 |
| | 90·7 | 94·5 | 104 | 45·2 | 39·2 | 87 | 45·5 | 41·5 | 91 |
| 2 | 91·2 | 93·0 | 102 | 46·1 | 37·5 | 81 | 45·1 | 39·0 | 87 |
| | 91·2 | — | — | 46·1 | 39·8 | 86 | 45·1 | 39·0 | 87 |

*Separation of arsenite from arsenate added to blood haemolysates (extraction procedure on aqueous dialysate).* Two experiments were performed as follows: To 150 ml. of a haemolysate were added measured amounts of arsenite and arsenate. Two 40 ml. samples were each dialyzed against 80 ml. of water in the ice-box for 24 hr., and the dialysates diluted to 100 ml. with distilled water; 40 ml. were used for total arsenic determination and the remaining 60 ml. for the xanthate extraction. For calculation of the results (Table 3), the assumption was made that the arsenite and arsenate were evenly distributed on either side of the dialysis membrane, and accordingly, the amounts of total arsenic, arsenite and arsenate found in the dialysate were multiplied by 3/2 to obtain the corresponding amounts in the original 40 ml. of haemolysate. Although this assumption may introduce some error in the actual figures for total arsenic, arsenite and arsenate, the ratio of the two latter should not be affected.

Table 4. *Recovery of arsenite and of arsenate added to blood haemolysates*

(Extraction procedure on trichloroacetic acid filtrates)

| | Arsenite (μg. As) | Arsenate (μg. As) |
|---|---|---|
| Total arsenic in laked blood | 226 | 262 |
| Total arsenic in trichloroacetic acid filtrate | 236 | 289 |
| Arsenite in trichloroacetic acid filtrate | 226 | 3 |
| Arsenate in trichloroacetic acid filtrate | 16 | 250 |

*Recovery of arsenite and of arsenate added to blood haemolysates (extraction procedure on trichloroacetic acid filtrates).* To a haemolysate prepared by diluting 15 ml. of blood with 65 ml. of distilled water, about 250 μg. As as arsenite or arsenate were added. Three 5 ml. samples were removed for total arsenic determinations, and 55 ml. were run slowly into 2 vol. of 20% trichloroacetic acid, made up to 250 ml. with distilled water and filtered. 20 ml. samples of the filtrate were used for determination of total arsenic, and 50 ml. samples for arsenite and arsenate separation. The results of these experiments are shown in Table 4, which records average figures for triplicate determinations.

*Separation of arsenite from arsenate added to urine dialysates.* Known amounts of arsenite and arsenate were added to 50 ml. portions of a dialysate of rabbit urine (1 vol. of urine against 10 vol. of distilled water), and the resulting solutions submitted to the xanthate procedure (Table 5).

*Recovery of arsenite from urine (extraction procedure on aqueous dialysate).* 15 ml. samples of rabbit urine, containing 30 μg. As as arsenite, were dialyzed against 60 ml. of water in the ice-box for 40 hr., and extracted as previously described. To obtain the results (Table 6), the experimentally determined values were multiplied by 5/4, it being assumed that there was an even distribution of arsenite and arsenate on both sides of the dialysis sac.

Table 5. *Separation of arsenite from arsenate added to urine dialysates*

| Arsenite | | | Arsenate | | |
|---|---|---|---|---|---|
| Added (μg. As) | Found (μg. As) | Recovery (%) | Added (μg. As) | Found (μg. As) | Recovery (%) |
| 20·0 | 19·6 | 98 | 20·1 | 22·2 | 110 |
| 20·0 | 18·2 | 91 | 20·1 | 22·3 | 111 |
| 40·0 | 38·3 | 96 | 40·3 | 39·6 | 98 |
| 40·0 | 34·2 | 86 | 40·3 | 43·2 | 107 |
| 120·0 | 111·7 | 93 | 80·6 | 83·3 | 103 |
| 240·0 | 241·2 | 100 | 120·8 | 125·8 | 104 |
| 80·0 | 82·8 | 104 | 282·0 | 282·1 | 100 |

*Recovery of arsenate from urine.* Approximately 450 μg. As (as arsenate) were added to 45 ml. of rabbit urine, which was dialyzed against 180 ml. of distilled water in the ice-box. After dilution of the dialysate to 200 ml., six 20 ml.

Table 6. *Recovery of arsenite from urine*

(Extraction procedure on aqueous dialysates)

| Arsenite added (μg. As) | Arsenite found (μg. As) | Arsenate found (μg. As) |
|---|---|---|
| 30·0 | 26·0 | 2·9 |
| 30·0 | 25·0 | 0·8 |
| 30·0 | 27·5 | 1·1 |
| 30·0 | 29·4 | 3·8 |

Table 7. *Recovery of arsenate from urine*

(Extraction procedure on aqueous dialysates)

| Total arsenic (μg. As) | Arsenite found (μg. As) | Arsenate found (μg. As) |
|---|---|---|
| 41·0 | Nil | 40·9 |
| | Nil | 38·7 |
| | 0·6 | 39·7 |
| 42·2 | 0·9 | 40·3 |
| | Nil | 41·9 |
| | Nil | 40·5 |

portions were used for xanthate extraction, and two 20 ml. samples for total arsenic determination (Table 7).

*Separation of arsenite from arsenate in urine.* Known amounts of arsenite and arsenate were added to rabbit urine and duplicate 5 ml. samples dialyzed against 60 ml. of distilled water in the ice-box for 46 hr. Table 8 gives the arsenite and arsenate values obtained by extracting the individual dialysates. For calculation of these results the small amount of arsenic remaining in the sacs—less than 10% of the total—was neglected (Table 8).

When the method is applied to biological fluids, it must always be borne in mind that arsenic-containing compounds other than arsenite and arsenate may be present. Any such compound soluble in carbon tetrachloride would appear in the arsenite fraction, irrespective of the valency of the arsenic. This difficulty may be overcome by carrying out preliminary extractions with carbon tetrachloride before extracting with the reagent.

When studying problems of metabolism by this method, it is necessary to assume that any arsenic compound which is water-soluble and dialyzable, or non-precipitable by trichloroacetic acid, and which is not extractable from acid solution by carbon tetrachloride, is either inorganic arsenite or arsenate. While this assumption is probably valid in studies with inorganic compounds of arsenic, it is obviously not justifiable in the case of administration of organic arsenicals.

Table 8. *Separation of arsenite from arsenate in urine*

(Extraction procedure on aqueous dialysates)

| Arsenite | | | Arsenate | | |
|---|---|---|---|---|---|
| Added (μg. As) | Found (μg. As) | Recovery (%) | Added (μg. As) | Found (μg. As) | Recovery (%) |
| 20·0 | 19·2 | 96 | 20·1 | 22·4 | 112 |
| 20·0 | 22·3 | 112 | 20·1 | 20·5 | 102 |

## DISCUSSION

By the method described above, it is possible to separate added inorganic arsenite from arsenate in simple solution, in blood haemolysates, and in urine with a reasonable degree of accuracy. They may be estimated in the separated fractions by any suitable arsenic method, the error involved in the xanthate extraction being probably less than that associated with the subsequent determination of arsenic. The method appears to be independent of the presence of those substances which would interfere with titrimetric, colorimetric or polarographic techniques.

## SUMMARY

1. A method is described for the quantitative separation of inorganic arsenite from arsenate in simple solution, in blood and in urine.

2. The arsenite is separated from arsenate by extraction with a carbon tetrachloride solution of sodium ethyl xanthate. Arsenate may be present in large excess.

3. The limitations of the method are discussed.

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# Glycogen

## 9. THE MAGNITUDE OF THE 'UNIT CHAINS' OF LIVER GLYCOGEN OF RABBITS SUPPLIED WITH GLUCOSE, FRUCTOSE AND SUCROSE

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End-group assay of glycogens from different sources has revealed that the 'unit chains' may be composed of either 12 or 18 glucose radicals. In the case of rabbit liver glycogen 18-radical specimens have been described by Bell (1936) after administration of galactose by mouth, and by Haworth, Hirst &

Isherwood (1937) for samples of commercial origin. The present paper describes the end-group assay (Haworth & Machemer, 1932; Hirst & Young, 1938) of glycogens isolated from livers of previously fasted rabbits subsequent to the administration of massive doses of glucose, fructose and sucrose. Fructose was given both by stomach-tube and by continuous intravenous injection; glucose was intravenously injected and sucrose given by stomach-tube alone. Investigation of a specimen of glycogen isolated from rabbit livers following continuous injection of glucose has already been reported (Bell, 1935, 1936); in this case a unit chain-length of 12 was reported.

## RESULTS

The results of our experiments, carried out on groups of animals, were clear-cut. Glycogen with 12 radicals was formed after the feeding of both glucose and fructose, and after the injection of fructose. Sucrose feeding, on the other hand, resulted in the formation of glycogen having a unit chain composed of 18 glucose radicals. This result should be contrasted with previous findings (Bell, 1935, 1936) on animals allowed to feed naturally on a diet of carrots augmented with sucrose. It seems possible in the light of our results that the 18-radical glycogen of commercial origin described by Haworth *et al.* (1937) may have been isolated from rabbits fasted and then fed massive doses of sucrose, this being a well-known method of stocking the liver with glycogen.

To summarize results up to the present, rabbit liver glycogen having 18 radicals composing the unit chain has now been found when both galactose and sucrose are fed to fasted animals. Feeding or intravenous injection of glucose and fructose, likewise to fasted animals, leads to formation of 12-radical unit chains. Feeding rabbits on a diet rich in sucrose therefore appears to have an effect different from that obtained when sucrose is given in concentrated solution by stomach-tube to fasted animals.

Interpretation of our results does not seem possible at the moment in view of our lack of knowledge of the mechanisms that underlie the formation of glycogen in the liver from substances other than glucose. Even with glucose our knowledge is incomplete, since the action of liver phosphorylase appears to produce a long, unbranched chain of glucose radicals analogous to amylose, whereas natural glycogen is composed of much shorter chains, aggregated in the manner Haworth and his colleagues have suggested and termed the 'laminated structure'.

During the course of this and similar investigations in this laboratory it became apparent that no rapid method was available for evaluating the properties of 2:3:4:6-tetramethyl- and 2:3:6-trimethyl-methylglucosides present in the mixed fractions encountered during end-group assays. One of us (J.S.D.B.) has devised a graphical method of analysis based on the following arguments:

Hirst & Young (1938) showed that, if the refractive indices of mixtures of the α- and β-isomers of certain methylated glucosides are plotted against their optical rotations, the straight lines shown in Fig. 1 are obtained.

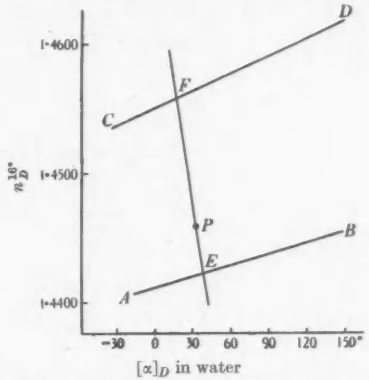


Fig. 1. Relationships between $[\alpha]_D$ (water) and $n_D^{16°}$ for mixtures of α- and β-methylglucosides of 2:3:6-tri- and 2:3:4:6-tetra-methylglucoses. For explanation see text.

*AB* represents all possible mixtures of 2:3:4:6-tetramethyl-α-methylglucoside with its β-isomer: *CD* represents the values for the two 2:3:6-trimethyl-methylglucosides. It will be seen that the two lines are almost parallel, and that both are finite. If it be assumed that a fraction obtained by distillation of the mixed glucosides (produced by hydrolysis of methylated glycogen, and believed to contain nothing but the four substances represented by the points *A*, *B*, *C* and *D*) has the properties represented by the point *P*, it is possible to make certain deductions as to its probable composition.

If any straight line *EPF* be drawn through *P*, then the fraction *P* can be expressed as a mixture of the two α-β-mixtures represented by *E* and *F*. In order to determine exactly the composition of a given fraction, the line *EPF* must be drawn in the right place.

It can be seen, however, that since *AB* and *CD* are almost parallel and since they are also finite, the limits of variation of the ratio *EP*/*PF* are narrow. If *AB* and *CD* were quite parallel the ratio would be constant. But *EP*/*PF* represents the ratio of tri- to tetra-methylglucosides in the fraction *P*, and is all that is required to be known. Consequently, if the variation possible in this ratio is sufficiently small, a suitably approximate answer to the problem of the composition can be obtained by graphical means.

The validity of this argument has been confirmed by algebraic methods.

## EXPERIMENTAL

### *Preparation of the glycogens*

(*a*) The sugars injected were administered by the method of Bridge & Noltie (1935). A 25% solution of glucose or fructose in normal saline was injected into the ear veins of

Table 1. *Summary of feeding and injection experiments*

| No. of animals in group | Body-weight (kg.) | | Sugar administered | | | | Weight of liver tissue | | Liver glycogen isolated | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total | Average | Injected | Fed | (g.) | (g./kg.) | (g.) | (g./kg.) | (g.) | (g./kg.) |
| 5 | 8·5 | 1·7 | Glucose | — | 156 | 18·3 | 415 | 4·88 | 26 | 6·25 |
| 8 | 16·0 | 2·0 | Fructose | — | 294 | 18·3 | 505 | 3·16 | 22 | 4·35 |
| 4 | 6·0 | 1·5 | — | Fructose | 130 | 21·6 | 325 | 5·4 | 21 | 6·46 |
| 4 | 5·6 | 1·4 | — | Sucrose | 130 | 23·2 | 280 | 5·0 | 20 | 7·15 |

Table 2. *Properties of the glycogen specimens and of their derivatives*

| Origin of specimen | $[\alpha]_D$ in water | Triacetyl glycogen | | Trimethyl glycogen | | |
|---|---|---|---|---|---|---|
| | | $[\alpha]_D$ in chloroform | Acetyl (%) | No. of treatments | $[\alpha]_D$ in chloroform | Methoxyl (%) |
| Glucose injection | +195° | +169° | 45 | 20 | +208° | 45·2 |
| Fructose injection | +199° | +167° | 44 | 8 | +210° | 45·2 |
| Fructose feeding | +193° | +167° | 44 | 12 | +202° | 45·3 |
| Sucrose feeding | +197° | +163° | 47 | 8 | +200° | 43·4 |

Table 3. *End-group assay on the specimens of glycogen*

| Fraction | Weight (g.) | $n_D^{16°}$ | $[\alpha]_D$ in water | Methoxyl (%) | Methylglucosides | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Tetramethyl | | Trimethyl | | Dimethyl | |
| | | | | | (%) | (g.) | (%) | (g.) | (%) | (g.) |
| A. Product from glucose-injected animals. 24·0 g. of methylated glycogen assayed | | | | | | | | | | |
| I | 1·364 | 1·4428 | +32·6° | 60·5 | (100) | 1·364 | — | — | — | — |
| II | 0·353 | 1·4439 | 72·0 | 60·0 | (100) | 0·353 | — | — | — | — |
| III | 3·668 | 1·4557 | 17·0 | 52·5 | (3) | 0·11 | (97) | 3·558 | — | — |
| IV | 10·844 | 1·4580 | 49·0 | 52·5 | — | — | (100) | 10·844 | — | — |
| V | 3·976 | 1·4586 | 63·1 | 52·5 | — | — | (100) | 3·976 | — | — |
| VI | 0·797 | 1·4597 | — | 51·2 | — | — | (91) | 0·725 | (9) | 0·072 |
| VII | 0·473 | — | — | 44·2 | — | — | (30) | 0·142 | (70) | 0·331 |
| VIII | 0·550 | 1·4720 | — | 41·8 | — | — | — | — | (100) | 0·550 |
| Residue (essentially derived from 2:3:6-trimethyl glucose), 0·40 g. Total pentamethyl glucose amounted to 1·872 g. With the addition of the usual 10% correction for experimental losses, a chain-length of 12 radicals is indicated. | | | | | | | | | | |
| B. Product from fructose-injected animals. 17·5 g. of methylated glycogen assayed | | | | | | | | | | |
| I | 0·641 | 1·4446 | +76·2° | 61·8 | (95·6) | 0·612 | (4·4) | 0·029 | — | — |
| II | 0·533 | 1·4461 | 95·5 | 60·6 | (89·1) | 0·474 | (10·9) | 0·059 | — | — |
| III | 2·844 | 1·4569 | 52·3 | 52·4 | (7·0) | 0·199 | (93) | 2·645 | — | — |
| IV | 5·006 | 1·4601 | 75·6 | 51·4 | — | — | (100) | 5·006 | — | — |
| V | 2·248 | 1·4601 | 122·0 | 50·2 | — | — | (79) | 1·776 | (21) | 0·472 |
| VI | 4·139 | 1·4737 | — | 42·0 | — | — | (10) | 0·414 | (90) | 3·725 |
| Residue (see A above), 0·92 g. Total pentamethyl glucose amounted to 1·285 g. With the usual 10% correction, the chain-length of this material is 11–12 radicals. | | | | | | | | | | |
| C. Product from fructose-fed animals. 6·01 g. of methylated glycogen assayed | | | | | | | | | | |
| I | 0·512 | 1·4463 | +52·0° | 59·0 | (76) | 0·389 | (24) | 0·122 | — | — |
| II | 0·685 | 1·4572 | 42·9 | 52·2 | (4) | 0·072 | (96) | 0·658 | — | — |
| III | 1·008 | 1·4586 | 63·8 | 51·5 | — | — | (100) | 1·008 | — | — |
| IV | 1·671 | — | 75·5 | 51·6 | — | — | (100) | 1·671 | — | — |
| V | 1·200 | — | — | 44·1 | — | — | (30) | 0·36 | (70) | 0·84 |
| Residue, 0·4 g. Total pentamethyl glucose amounted to 0·4 g. With the 10% correction the chain-length of this material is 11–12 radicals. | | | | | | | | | | |
| D. Product from sucrose-fed animals. 26·0 g. of methylated glycogen assayed | | | | | | | | | | |
| I | 0·912 | 1·4435 | +33·6° | 61·1 | (100) | 0·912 | — | — | — | — |
| II | 2·833 | 1·4543 | 15·6 | 53·0 | (14) | 0·400 | (86) | 2·433 | — | — |
| III | 2·660 | 1·4570 | 27·7 | 51·8 | — | — | (100) | 2·660 | — | — |
| IV | 10·000 | 1·4586 | 63·4 | 52·0 | — | — | (100) | 10·000 | — | — |
| V | 2·902 | 1·4630 | — | — | — | — | | Not analyzed | | |
| VI | 4·400 | 1·4700 | — | — | — | — | | Not analyzed | | |
| VII | 0·76 | 1·4758 | — | 40·2 | — | — | — | — | (100) | 0·76 |
| Residue, 0·4 g. Total pentamethyl glucose amounted to 1·312 g. With the 10% correction the chain-length of this material is 18–19 radicals. | | | | | | | | | | |

normal rabbits, previously fasted for 36 hr., at an average rate of 4·6 g. of sugar/kg. body weight/hr. for a period of 4 hr. The animals were then anaesthetized by injection of urethane into the ear vein, the livers removed and the glycogen isolated by the Pflüger technique.

(*b*) Rabbits, approximately 1·5 kg. in weight, were fasted for 36 hr., and 130 ml. of a 25% solution of fructose or sucrose were administered by stomach-tube. After 4 hr. the liver glycogen was isolated as for the injected animals.

(*c*) Purification of the samples was effected by the method of Bell & Young (1934). Data relating to the preparation of the specimens of glycogen obtained are summarized in Table 1. It will be seen that fructose administered by injection appears to form less glycogen than fructose given by mouth.

*End-group assay*

The specimens were acetylated and then subjected to simultaneous deacetylation and methylation, followed by fractional distillation of the products of methanolysis as described in earlier parts of this series. The results are given in Tables 2 and 3.

## SUMMARY

1. Three sugars were administered to fasting rabbits and the unit chain-length of the liver glycogen was determined.

2. Glycogen with a chain-length of 12 glucose radicals was produced by feeding fructose and by the continuous intravenous injection of fructose or of glucose.

3. Glycogen with a chain-length of 18 radicals was produced by feeding sucrose.

4. Fructose administered by injection appears to form less glycogen than when given by mouth.

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